CMLS, Cell. Mol. Life Sci. 56 (1999) 742–754
1420-682X/99/100742-13 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Antibiotic resistance in microbes

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Abstract. The treatment of infectious disease is compromised by the development of antibiotic-resistant strains of microbial pathogens. A variety of biochemical processes are involved that may keep antibiotics out of the cell, alter the target of the drug, or disable the antibiotic. Studies have shown that resistance determinants arise by either of two genetic mechanisms: mutation and acquisition. Antibiotic resistance genes can be disseminated among bacterial populations by several processes, but principally by conjugation. Thus the overall problem of antibiotic resistance is one of genetic ecology and a better understanding of the contributing parameters is necessary to devise rational approaches to reduce the development and spread of antibiotic resistance and so avoid a critical situation in therapy—a return to a pre-antibiotic era.

Key words. Horizontal gene transfer; integron; acquisition; mutation; ecology.

Introduction

The story of antibiotics and antibiotic resistance is of recent origin, since antibiotics have been used for the treatment of infectious diseases in humans and also for a number of non-human applications (agriculture, animal husbandry, and aquaculture) during the last half century only. This was an enormously successful period in medical history during which most of the major diseases of humankind were more or less brought under control (at least in the industrial nations of the world). This situation is now threatened by the increasing incidence of antibiotic resistance [1]. The microbes that were once susceptible to antibiotics are becoming more and more difficult to treat. The objective of this short review is to describe the ways by which microbes have become resistant: essentially this is due to the extraordinary genetic flexibility of bacteria, a factor which continues to challenge microbiologists. Nonetheless, there is currently a reasonable state of understanding with respect to the development of antibiotic resistance, the ways in which it may be disseminated and the biochemical mechanisms responsible. Not surprisingly, bacteria can employ many different processes to establish resistance to infectious disease therapy and it is likely that the complete story is not yet revealed.

The roles of low-molecular-weight biologically active components in microbial ecology are poorly understood [2]. But it is important to recognize that the antibiotics introduced in the period 1940–1950 were not novel biological agents but common natural microbial products whose useful antibiotic activity had been discovered by laboratory screening in the pharmaceutical industry. Subsequently, many semi-synthetic derivatives were made on these natural structural scaffolds and employed therapeutically; some antibiotics, however, are novel synthetic molecules which have no known natural counterpart [3]. What was new and different in the 1950s was the nature and magnitude of their uses. Enormous quantities of natural products with demonstrated and tested therapeutic activities (the antibiotics) were manufactured for human use by a new generation of pharmaceutical companies and released into the biosphere; being toxic agents they created a catastrophic situation for terrestrial bacteria, with significant knock-on effects on the microbial ecology in many different ecosystems (it has been estimated that more than a million metric tons of antibiotics have been released

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into the biosphere during the last 50 years) [4]. What actually happened in the early years following the widespread use of antibiotics is a matter of speculation, since microbial ecology was a fledgling science at the time and retrospective analyses are difficult to perform; some bacterial species in localized microbial populations may well have been eliminated, but there is no doubt that significant variations in the microbial population and extensive genetic modification (the acquisition of resistance genes) must have taken place whenever antibiotics were used. The subsequent half-century of antibiotic use has undoubtedly influenced all aspects of microbial genetic ecology through mutation, selection, and the flux of genetic information (horizontal gene transfer) between microbes; this has been a period of strong evolutionary pressure and extensive selection. In essence, a large, uncontrolled microbiological experiment has taken place in the second half of the 20th century, with only the cultivable survivors available for investigation. It is possible to study this flux by analyzing resistance genes in microbial populations, and such retrospective analyses have provided substantial information on mechanisms of resistance, their origins, acquisition, and dissemination. Unfortunately, the exact details of these processes still escape us, because they involved large and uncharacterized microbial populations and the intermediate steps in events such as gene transfer cannot be modelled in the laboratory.

The problem: development of antibiotic resistance

With only rare exceptions, antibiotic resistance in bacterial pathogens was identified very soon after the introduction of antibiotics into clinical practice. Was this resistance due to mutation? Certainly in a few instances (for example, streptomycin resistance in *Mycobacterium tuberculosis*), mutations leading to clinically significant levels of resistance occurred, and it is very likely that mutations leading to low levels of resistance to antibiotics were frequent in other species and were an initial step in the development of the high-level-resistance strains found today [5]. However, in the majority of cases, acquisition from exogenous (and still largely unidentified) sources was the primary mechanism by which bacteria obtained genes encoding resistance to antibiotics [6]. Since only the infection-related strains were subsequently identified and studied, the extent and change of antibiotic susceptibility in overall microbial populations could not be analyzed. Even today, the demographics of populations under antibiotic stress are little understood because information on the nature of microbial populations in general is lacking. Horizontal gene transfer played a primary role in the development and dissemination of antibiotic resistance genes and it would be of great interest to follow the course of this gene trafficking from the time of first antibiotic exposure. Did resistance develop in many species or in only a limited number before being mobilized and transferred to the pathogens that were subsequently identified as antibiotic resistant? How many intermediate hosts were required? This is not known. However, it is to be hoped that when the next completely novel antibiotic (structural entity) is introduced into human clinical practice, appropriate population analyses and elucidation of the route(s) by which antibiotic resistance is established in the community will be carried out. It is important to realize that the use of an antibiotic affects *all* members of the resident microflora and not simply the targeted pathogen(s). Antibiotics, like any xenobiotic, act upon the entire population exposed, cultivable and non-cultivable.

Existing evidence strongly suggests that prior to the introduction and use of antibiotics, antibiotic-resistant microbes were absent from human or animal flora; this comes from studies of pre-antibiotic bacterial populations [7] and also from analysis of the development of bacterial resistance to antibiotics such as tetracycline following their introduction into the environment (fig. 1). In all cases studied there is a direct correlation over time between antibiotic use and the increase in proportion of resistant to non-resistant strains. The dramatic appearance of antibiotic resistance in pathogenic *Shigella* sp. during epidemics of intestinal infections in Japan in the 1950s is yet another example of the rapid response of bacteria to the threat of antibiotic use [8]; in

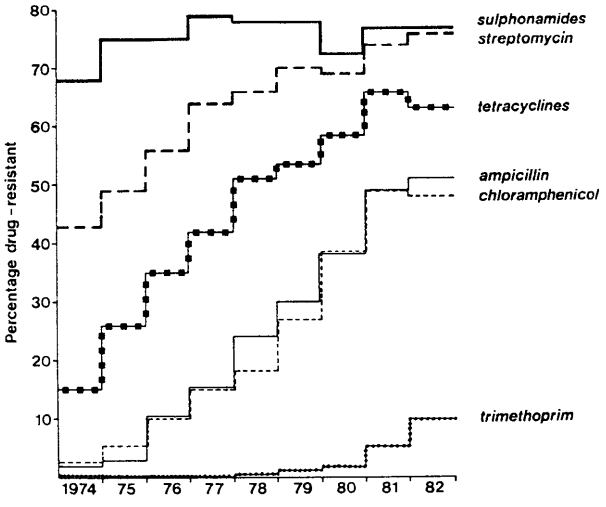

Figure 1. Development of antibiotic resistance in *Shigella* spp. in the United Kingdom 1974–1982. The increases follow patterns of antibiotic use during this period.

Table 1. Biochemical mechanisms of antibiotic resistance and their genetic determinants.

Mechanism	Examples	Genetic determinants	
		mutation	gene acquisition
Reduced permeability	aminoglycocides	+	+
Pro-drug not activated	isoniazid	+	
Active efflux	tetracycline fluoroquinolones		+
Alteration of drug target	erythromycin		+
	fluoroquinolones	+	
	rifampicin	+	
	tetracycline		+
Inactivation of drug	aminoglycosides		+
	chloramphenicol		+
	beta-lactams	+	+
	rifampicin	(+)	(+)
By-pass inhibited step	sulfonamides		+
	trimethoprim		+
Immunity protein	bleomycin		+
Amplification of target	trimethoprim	+	
	sulfonamides	+	
Sequestration of drug	beta-lactams	+	+

this case, the increase was clearly linked to the appearance and dissemination of plasmids encoding antibiotic resistance (R plasmids). Conversely, a number of studies have shown that the incidence of antibiotic resistance is often reduced when the use of antibiotics is restricted [9–11]. Unfortunately, it never disappears!

Upwards of a dozen different biochemical mechanisms of resistance have been characterized to date (table 1); these are encoded by hundreds of different genes, many identified as classes of related allelic determinants. The bacterial population has essentially stockpiled a considerable armamentarium of genetic defenses against antibiotics. Definitive information concerning the origins of these resistance genes (where did they come from and how did they move to where they are?) is hard to come by but, as discussed later, in a few cases there is now significant evidence as to the sources and mechanisms of their acquisition. The overall problem is one of microbial genetic ecology and the effects of a variety of strong selective stresses that have determined the make-up and use of bacterial gene pools [12]. Not surprisingly, the majority of antibiotic resistance genes are carried by plasmids, transposons, and other elements capable of inter-generic and inter-specific mobility.

Origins of antibiotic resistance genes

In recent years, there has been much discussion about the role of pathogenicity islands (PAIs) in the increasing virulence of bacterial clinical isolates. PAIs are clusters of genes providing those components necessary for the establishment and survival of the pathogen in the hostile environment of the infected host [13, 14]. Simply put, the difference between a pathogenic and a non-pathogenic *Escherichia coli* strain is the presence of extra genes in the form of PAIs in the pathogen. Likewise, antibiotic resistance genes are found in clusters that group different determinants of different origin (as shown by variations in G + C content and codon usage) at single sites on the bacterial chromosome. Such clustering has been recognized for some time and most likely reflects the roles of transposons, integrons, and plasmids as vehicles in the transport of resistance genes. Interestingly, in a number of cases, such resistance clusters are also associated with virulence determinants, i.e., the same plasmid encodes both functions [15]. The conclusion is that resistance genes are 'foreign' to their bacterial hosts and an obvious question is where do they come from? A number of plausible sources of resistance determinants have been proposed:

1) housekeeping genes (e.g., alternative substrates, mutation to new substrate recognition)
2) antibiotic-producing microbes (self-protection)
3) 'natural' resistance genes in soil communities

However, no single origin has been identified definitively. Nonetheless, as described below, in a few cases, a direct (single transfer) relationship can be established between the resistance gene(s) found in clinical isolates and their putative source organisms.

Resistance to the β-lactam antibiotics in *Neisseria* and *Streptococcus* sp. is due to the development of altered

penicillin-binding proteins (PBPs) with a decreased affinity for β-lactam antibiotics. Comparisons of the PBP genes from resistant and sensitive strains have shown that resistant PBPs have multiple gene segment replacements leading to mosaic structures. In most cases, the new gene segments were highly similar or identical to the corresponding part of the PBP gene from a closely related commensal species [16–22]. For example, in penicillin-resistant *Neisseria meningitis*, the *penA* gene contains two segments which show 22% divergence from the *penA* allele found in penicillin-sensitive strains, but which are identical to the corresponding regions from the *penA* gene of *N. flavescens*, a commensal species [23]. The fact that more than 30 different mosaic genes have been found in 78 isolates of penicillin-resistant *N. meningitis* exemplifies the extent to which this mechanism is common in naturally transformable species [24, 25].

Resistance to sulfonamide drugs is due to either the development or the acquisition of an altered dihydropteroate synthase (DHPS) with a decreased affinity for the inhibitor. In *N. meningitis*, *Streptococcus pneumoniae* and *S. pyogenes*, sulfonamide resistance is mediated by mutations in the chromosomal DHPS gene, *folP*. Comparisons of *folP* from resistant and sensitive strains have shown that in resistant isolates, the *folP* genes diverged by 8–14% from their counterparts in sensitive isolates [26–29], some of them having a mosaic structure [28]. Notably, one *N. meningitis*-resistant isolate had a mosaic *folP* gene whose central segment was identical to the corresponding segment of a *N. gonorrhoeae* strain [26]. As found for the mosaic *penA* genes, the number of different mosaic *folP* genes suggests that resistance has arisen independently on many occasions in these species.

However, in the majority of cases, the evidence permitting identification of antibiotic resistance genes is largely circumstantial and comes mostly from comparative biochemical and molecular studies; in several instances, the closeness of the nucleotide sequences indicates a strong relationship. One of the best examples is that of the gene cluster responsible for methicillin resistance (the *mec* cluster) found in *Staphylococcus aureus*, which was first identified in the early 1960s (shortly after the introduction of the new drug) and which has had a profound effect on the treatment of infection by this organism. Methicillin-resistant *S. aureus* (MRSA) is found worldwide and has been responsible for numerous serious hospital outbreaks that have proved difficult to treat. While MRSA refers to methicillin resistance, it has been found that such strains often carry resistance determinants for other antibiotics. Work from the group of Tomasz indicates that these determinants may have originated in *Staphylococcus sciuri* [30]. Similarly, the aminoglycoside acetyltransferase (*aac*) genes found in the enteric bacteria appear likely to have evolved from the chromosomal genes of other species of Gram-negative bacteria [31] as a result of horizontal gene transfer and mutation processes. Interestingly, the *aac* genes have been shown to be members of a large protein N-acetyltransferase family [32]. Other aminoglycoside acetyltransferases have significant primary sequence similarities to the acetyltransferases found in streptomycetes, and this is also the case for the aminoglycoside phosphotransferases (APHs), which are members of the protein kinase superfamily. In the latter case, the similarity extends to the three-dimensional structures of the APH proteins and other kinases [33].

The notion that the determinants encoding resistance to aminoglycosides, macrolides, tetracycline, chloramphenicol, glycopeptides, and other antibiotics originated in the organisms producing these secondary metabolites (especially streptomycetes) is strongly supported by studies of biochemical and nucleic acid sequence similarities of the genes and their products (table 2). The recent work of Wright and colleagues [34, 35] in characterizing resistance to the glycopeptides provides an ex-

Table 2. Antibiotic resistance gene families and their sources.

Resistance gene	Producer	Housekeeping	3D	Superfamily
aacB	+	+		
aacA	+	+		Protein *N*-acyltransferase
aacC	+	?	√	
aphA	+	?	√	Protein kinase
aadA	?	?	√	NMP transferase
bla	+	+	√	Serine protease
cat	?	?	√	X-*O*-acyltransferase
vanA,B	+	?		
erm	+	–	–	RNA methylase
tet	+	–		RND transporters, G proteins
sul	–	?	√	DHPS
tmp	–	?	√	DHFR

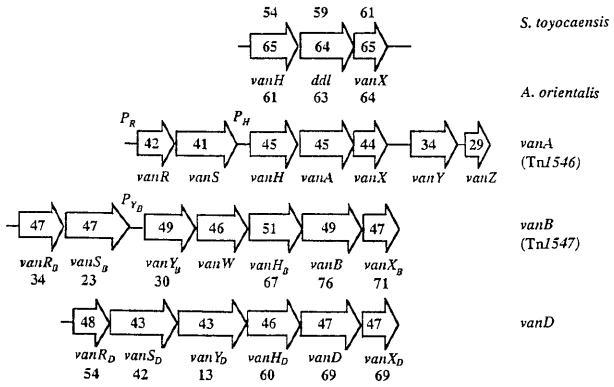

Figure 2. The *vanH*, *A*, *X* cluster of antibiotic-resistant enterococci compared with those identified in glycopeptide-producing actinomycetes. The numbers in the arrows indicate % G + C content and numbers between the clusters indicate % amino acid identity compared to the *vanA* cluster.

cellent example of this relationship between producer and clinical isolate (or an extraordinary coincidence!). Glycopeptide resistance in the enterococci is due to a cluster of genes that encode the synthesis of a novel cell envelope component, D-ala-D-lac that replaces the D-ala-D-ala moiety normally present. The replacement of D-ala by D-lac results in a 1000-fold reduction in glycopeptide binding, which explains the consequent high level of resistance to the antibiotics vancomycin and teicoplanin [36]. Three genes are required for the synthesis of D-ala-D-lac, and nucleotide sequence comparisons show a striking similarity between the *vanH*, *A*, and *X* genes of enterococci and the related clusters found in *Streptomyces toyocaensis* and *Amycolatopsis orientalis*, the two organisms producing glycopeptide antibiotics (fig. 2). This similarity extends to the structures of intergenic sequences in the various clusters, where the *vanH* and *vanA* genes and the actinomycete analogues are all translationally coupled through overlapping reading frames!

An example of an antibiotic resistance gene of unusual 'parenthood' is provided by mupirocin (pseudomonic acid), an antibiotic employed frequently in the treatment of topical infections caused by Gram-positive bacteria. Resistance to mupirocin is due to the presence of an altered isoleucyl tRNA synthase that may have originated in eukaryotes and was only recently acquired and transferred within *S. aureus* strains [37]. The molecular details of this unusual acquisition remain to be ascertained. It should be emphasized that in none of the cases described above has direct gene transfer been demonstrated between the source organism and the pathogen. However, as mentioned earlier, these are almost certainly multi-component transfer processes that take place within mixed bacterial populations.

With respect to origins of resistance, it should be noted that long before the use of antibiotics, a variety of mercury compounds were used as disinfectants, as were quaternary ammonium compounds. The significance of the pre-antibiotic use of such agents is not easy to assess, but it has been observed that genetic determinants for resistance to a variety of detergents are linked to genes for antibiotic resistance [38]. In some cases, the same biochemical mechanism (efflux) is known to influence the susceptibility of bacteria to both antibiotics and detergents.

Mutation

One must not underestimate the role played by spontaneous mutation in the development of resistance to antibiotics. On the one hand, there is the fact that the first significant resistant pathogen that influenced the use of a drug (streptomycin-resistant *M. tuberculosis*) developed resistance as the result of mutations affecting the target site of the antibiotic. Many other instances of clinically important resistance due uniquely to mutation have been identified, most recently the widespread development of resistance to the fluoroquinolones in both Gram-negative and Gram-positive bacteria, which is due principally to host mutations that render the drug target less susceptible to binding [39]. Nonetheless, mutation is more the exception than the rule, since the genetic basis of most antibiotic resistance among clinically significant bacteria is due to the dual process of gene acquisition and horizontal transfer. The question is, why does mutation play an important role in some cases and not others? This is difficult to answer and may involve various factors, such as the nature of the pathogen, the treatment regime and the biochemical nature of antibiotic action. Recent work from Björkman et al. [40] sheds additional light on this question. These authors observed that most *Salmonella typhimurium* mutants resistant to streptomycin, rifampicin, and nalidixic acid are avirulent in mice, which suggests that such mutants would be spontaneously eliminated from the host. Although it is not known whether acquired resistance genes have an effect on virulence in the same strains, it is notable that such resistant mutants have not been encountered in resistant clinical isolates of *S. typhimurium*. What is interesting is that a small proportion of the antibiotic-resistant mutants survived in the host and were found to have acquired compensatory mutations that restored virulence while not affecting the resistance phenotype. Additional studies are required to see if such double mutants are found in human infections with bacterial pathogens.

In the case of *M. tuberculosis*, no exogenously acquired resistance gene has been identified and plasmids have yet to be characterized in this species [41]. Despite the fact that mobile genetic elements such as insertion elements are common in mycobacteria, no compound transposons are found in this pathogen. Thus, the generation of the multi-drug resistant *Mycobacterium* strains that are so feared by physician and patient alike requires a series of independent mutations [42]. Since it has been shown that resistance arises in successive mutational steps, for a strain resistant to four drugs the frequency of appearance would be greater than 1 in 10^{25}! Similarly, in the case of fluoroquinolone resistance in Gram-positive or -negative bacteria, while the frequency of finding such target mutations in the laboratory is relatively low, in clinical practice, resistant strains appear during the course of patient treatment. These are frequently target alterations, but enhanced efflux systems are common in some genera [39]. Since the fluoroquinolones are completely synthetic agents with no known natural counterparts, the acquisition of dominant plasmid-encoded resistance determinants would not be anticipated; mutation is therefore a predictable process for the clinical development of resistance to this class of antibiotics. Unexpectedly, plasmid-encoded resistance to fluoroquinolones has been identified recently, but the mechanisms have not yet been elucidated [43]. We note that acquired resistance to trimethoprim and sulfonamides, which are both synthetic drugs, is almost exclusively due to plasmid-encoded resistance genes!

Two other important roles of mutation can be identified in the evolution of antibiotic resistance determinants. First, it is likely that hypermutable strains and mutator genes have a significant influence in the process of horizontal gene transfer. Extensive tailoring and adaptation of the resistance genes takes place during the passage from source organism to clinical isolates possessing different G + C composition, codon usage, and gene expression systems. How does this modification occur? As has been mentioned previously, intermediate transfer hosts certainly play a role in this process, but they have not been identified—or even looked for; only the 'final' resistant isolate is identified by the clinical microbiologist. Recent studies have shown that many natural bacterial isolates, particularly pathogenic strains, may be hyper-mutagenic because they carry one or more mutator genes (*mutS*, *mutL*, *mutT*) [44, 45]: in some cases the mutator genes involved, such as *mutT*, would favor the conversion of high G + C sequences and thus readily contribute to adaptation of codon usage patterns to new cellular environments [46]. Unfortunately, the natural incidence of mutator strains in environmental microbes and their possible roles in the tailoring of antibiotic resistance genes (or any horizontally transferred determinants, such as biodegradation clusters) has not been examined systematically in natural populations and their importance is not yet established.

Second, the development of extended-spectrum β-lactamases (which engender resistance to newer synthetic derivatives of penicillins and cephalosporins) occurs by protein engineering in vivo. Point mutations that change critical amino acids within the β-lactamases have been characterized; these lead to alterations in enzyme/substrate recognition and have generated a huge family of genetically engineered resistance genes. Thus a critical juxtaposition of mutation and gene transfer is well established in the evolution of extended-spectrum β-lactamases [47].

Finally, it must be re-emphasized that bacteria are not single isolates in the environment. The concurrent roles of the different members of complex bacterial communities should not be underestimated. The structure and properties of such communities are not understood, but there is recognition of the important roles that biofilms play in horizontal gene transfer and other social activities of bacteria. In addition, as will be mentioned later, the genetic ecology of antibiotic resistance is inextricably linked to the ecology of microbial populations in humans, animals, and plants, and it is necessary to consider this in a 'holistic' manner.

Mechanisms of acquisition (resistance gene capture)

One of the most significant aspects of the development of antibiotic resistance in bacteria is the tandem assembling of resistance genes within single, mobile genetic elements to generate multi-drug-resistance clusters. This has many implications.

Multi-drug resistance includes the following scenarios:

1) A single plasmid or transposon encodes resistance to several different antibiotics; e.g., clusters.

2) A single gene encodes a biochemical mechanism that engenders resistance to a class of related antibiotics; e.g., *erm* triggers resistance to the macrolides; *aac*, *aph*, *ant* to the aminoglycosides, and *bla* to the β-lactams.

3) A single resistance gene encodes resistance to a group of structurally unrelated antibiotics, e.g., *aad* determines resistance to both streptomycin and spectinomycin, or various non-specific efflux systems.

4) A non-antibiotic, such as a disinfectant or organic salt (e.g., mercury), selects for the establishment of genetically linked antibiotic resistance genes.

5) Mutations occur in multiple, independent target genes (as in *M. tuberculosis*).

The most common mechanism by which tandem arrays of antibiotic resistance genes are assembled in the Enterobacteriaceae is gene capture by integrons: many

resistance determinants are known to be carried by such elements (table 3). The process has been dissected genetically and biochemically by various researchers [48–50]. All known integrons are composed of three essential elements: a gene encoding an integrase, a primary recombination site, and a strong promoter. The integron-associated integrases belong to the site-specific recombinase family and are able to recombine discrete units of DNA, now known as gene cassettes, providing them with a promoter for their expression. Most of the resistance cassettes contain only a single resistance gene associated with a specific recombination sequence known as the 59-base-pair (bp) elements or *attC*. An outline of the integron-integrase acquisition process is shown in figure 3. While this scheme provides an overall explanation of the process, many important details need to be elucidated. For example, from where do the integrases and the 59-bp elements which are essential for the recognition and integration of the captured gene cassettes originate? Are they derived from bacteriophages? What are the 59-bp elements and how do they become attached to the individual open reading frames (ORFs) in order to form the linear or circular gene cassettes?

Recent work by Mazel et al. [51] has shed some light on these matters. Studies of relationships between integrons and *Vibrio cholerae* clusters (VCRs) have demonstrated striking structural similarities. The two compound genetic elements are shown in figure 4 and the commonalities are obvious. Both the VCR elements and antibiotic-resistant integrons possess specific integrases that are responsible for the insertion of coding sequences (ORFs) into a unique chromosomal recombination site, leading to the formation of tandem arrays of genes (albeit of unidentified function in *V. cholerae*). Furthermore, the fact that gene-VCR structures are substrates for the integrase of antibiotic-resistant integrons shows that they are functionally identical to integron cassettes. However, in the case of *V. cholerae*, the cluster of VCR cassettes represents 60–100 unidentified genes and occupies approximately 4% of the 2.5-Mb genome [52]. It is not yet established whether this new type of integron, the chromosomal super-integron, is harbored on a mobile structure, like those propagating the three classes of antibiotic-resistant integrons. Preliminary studies indicate that integrase-related structures with the same general characteristics are found in a number of different *Vibrio* sp. The two *Vibrio* integrases so far identified are related to each other and form a class related to, but distinct from, IntI2 (of class 2 integrons) (fig. 5). The nature of the integrase-associated primary recombination sites has not been demonstrated in all cases, nor is it known if they are integrase specific. The *Vibrio* super-integrons differ from the integrons described earlier in that (i) a number of the ORFs possess their own promoter, (ii) an identical repeat (VCR) is associated with each ORF, (iii) they are related principally to virulence functions and no well-defined antibiotic resistance genes are found in the clusters, although it should be noted that the *blaP3* and *dfrVI6* cassettes of integrons are VCR associated. These structures reveal a significant relationship between resistance and virulence genes and imply that the selective pressure of antibiotic use could have promoted the dissemination of pathogenicity determinants in the development of new pathogens.

The global release of antibiotics over the past 50 years did more than apply pressure for the selection of protective mechanisms (resistance genes) in the microbial population; the horizontal transfer of genes unrelated to resistance must have taken place simultaneously. We consider it likely that extensive movement of virulence determinants occurred at random during dissemination of resistance genes. It is intriguing that many *Vibrio* sp. obviously possess a large number of clustered genes of unknown function that have been captured by a highly efficient and specific process: a veritable 'candy store' of genetic information that is readily accessible to other *Vibrio* and related bacterial species. Detailed analysis of the ORFs is in process for different species; the results so far confirm the diversity of sequences present. If each *Vibrio* sp. proves to have VCR clusters of hundreds of unidentified genes as in *V. cholerae*, a treasure trove will be available for functional genomic studies, since these clusters clearly represent sequences that are not essential for host bacterial growth and maintenance under normal conditions. Or they could be junk! It is clear that the number of different super-integrons may be large, each classified according to its associated site-specific integrase. If they are not mobile by themselves, they may provide an inexhaustible source of both cassettes and integrases for the building of other mobile integrons, such as those implicated in the dissemination of antibiotic resistance. In addition to its role in resistance and pathogenicity, integron-driven gene capture is also likely to be important in the more general process of horizontal (lateral) gene transfer in the evolution of the bacterial genome. Such functional tandem arrays of independent genes have been identified so far only in enteric bacteria (although integrons have been found recently in the Gram-positive *Corynebacterium glutamicum* [53] and *M. fortuitum* [54], and we note that tandem arrays of resistance genes have been characterised in Gram-positive bacteria such as *S. aureus* [55]; in the latter case, no associated intergenic structural element or integrase can be identified in these clusters.

Table 3. Gene cassettes encoding antibiotic resistance genes found in integrons[a].

Gene cassette[b]	Protein[c]	Activity	Accession No.[d]
Resistance to β-lactams			
blaP1	CARB-2 (PSE-1), CARB-3, PSE-4	β-lactamase	Z18955
blaP2		β-lactamase	D13210
blaP3	CARB-4	β-lactamase	U14749
bla_{IMP}	IMP-1	β-lactamase	D50438
$bla_{CEF\text{-}1}$		β-lactamase	AF078527
oxa1	OXA-1, OXA-8, OXA-4	β-lactamase	J02967
oxa2	OXA-2, OXA-15	β-lactamase	M95287
oxa3	OXA-3	β-lactamase	L07945
oxa5	OXA-5	β-lactamase	X58272
oxa7	OXA-7	β-lactamase	X75562
oxa9	OXA-9	β-lactamase	M55547
oxa10	OXA-10 (PSE-2), OXA-11, OXA-14	β-lactamase	U37105
oxa20	OXA-20	β-lactamase	AF024602
Resistance to aminoglycosides			
To streptomycin and spectinomycin			
aadA1a	AAD(3″)	aminoglycoside (3″) adenylyltransferase	X12870
aadA1b	AAD(3″)	aminoglycoside (3″) adenylyltransferase	M95287
aadA2	AAD(3″)	aminoglycoside (3″) adenylyltransferase	X68227
To other aminoglycosides			
aadB	AAD(2″)	aminoglycoside (2″) adenylyltransferase	L06418
aacA1	AAC(6′)-Ia	aminoglycoside (6′) acetyltransferase	M18967
aacA	AAC(6′)-Ip	aminoglycoside (6′) acetyltransferase	Z54241
aacA	AAC(6′)-Iq	aminoglycoside (6′) acetyltransferase	AF047556
aacA4	AAC(6′)-Ib, AAC(6′)IIc	aminoglycoside (6′) acetyltransferase	M55547
aacA	AAC(6′)-IIa	aminoglycoside (6′) acetyltransferase	M29695
aacA	AAC(6′)-IIb	aminoglycoside (6′) acetyltransferase	L06163
aacA (orfB)	AAC(6′)-Id	aminoglycoside (6′) acetyltransferase	X12618
aacA7	AAC(6′)-Il	aminoglycoside (6′) acetyltransferase	U13880
aacC1	AAC(3)-Ia	aminoglycoside (3) acetyltransferase	X15852
aacC	AAC(3)-Ib	aminoglycoside (3) acetyltransferase	L06157
Resistance to chloramphenicol			
catB2	CATB2	chloramphenicol acetyltransferase	M80188
catB3	CATB3	chloramphenicol acetyltransferase	U13880
catB5	CATB5	chloramphenicol acetyltransferase	X82455
catB6	CATB6	chloramphenicol acetyltransferase	X98393
cmlA	CmlA	chloramphenicol exporter	U12338
cmlA2	CmlA2	chloramphenicol exporter	AF034958
Resistance to trimethoprim			
dfrA1	DHFRIa	dihydrofolate reductase (classA)	X00926
dfrA5	DHFRV	dihydrofolate reductase (classA)	X12868
dfrA6	DHFRVI	dihydrofolate reductase (classA)	Z86002
dfrA7	DHFRVII	dihydrofolate reductase (classA)	X58425
dfrA12	DHFRXII	dihydrofolate reductase (classA)	Z21672
dfrA13	DHFRXIII	dihydrofolate reductase (classA)	Z50802
dfrA14	DHFRIb	dihydrofolate reductase (classA)	S76821
dfrA15	DHFRXV	dihydrofolate reductase (classA)	Z83311
dfrA16	DHFRXVI	dihydrofolate reductase (classA)	AF077008
dfrB1	DHFRIIa	dihydrofolate reductase (classB)	U36276
dfrB2	DHFRIIb	dihydrofolate reductase (classB)	J01773
dfrB3	DHFRIIc	dihydrofolate reductase (classB)	X72585
Resistance to streptothricin			
sat	SAT-2	streptothricin acetyltransferase	X15995
Resistance to rifampin			
arr2	ARR -2	ADP-ribosyl transferase	AF078527
Resistance to erythromycin			
ereA2	EreA2	erythromycin esterase	AF099140
Resistance to antiseptics and disinfectants			
qacE	QacE	quaternary ammonium compound exporter	X72585
qacE2	QacE2	quaternary ammonium compound exporter	AJ223604
qacF	QacF	quaternary ammonium compound exporter	AF034958

[a] This is an updated version of the compilation of antibiotic resistance cassettes originally published by Hall and Collis [61].
[b] Cassettes are named after the gene they encode. In some cases, the genes have not previously been assigned names or the gene nomenclature used here differs from that found in the original publications or sequence entries. As an agreed numbering system for the *aacA* and *aacC* genes is not available, several of the genes are not numbered.
[c] Where more than one protein name is listed, the nucleotide sequences differ at fewer than five positions or are identical (for names in parentheses).
[d] GenBank and EMBL database accession numbers. In several cases, the cassettes are either not identified or improperly identified in the database entries.

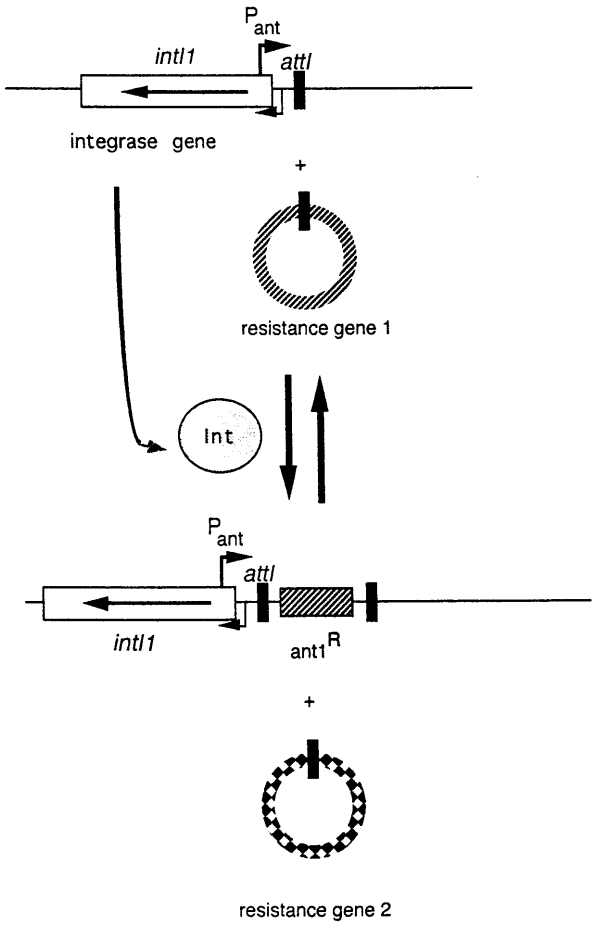

Figure 3. Outline of the process by which circular antibiotic resistance gene cassettes are inserted into a specific attachment sequence (*attI*) and so placed in a linear fashion in a genome, downstream of a functional promoter element (P_{ant}).

The big picture—the ecology of antibiotic-resistant bacteria

We have focused discussion up to now on the genetic ecology of antibiotic resistance: the acquisition, dissemination, and organization of resistance *genes* within bacterial communities. However, a wide variety of antibiotic-resistant bacteria are themselves widely distributed in different ecological niches. The spread of these resistant organisms is an issue that is independent of the horizontal transfer of resistance genes; obviously both contribute to the overall problem. Antibiotic-resistant strains are associated with many different environments and the following are important factors that contribute to the macro-ecology of such strains:

1) health care institutions and the maintenance and transmission of resistant strains within the community (e.g., food and domestic animals);

2) the dissemination of antibiotic-resistant bacteria in animals and their different environments;

3) global transfer of antibiotic-resistant strains (pathogens and non-pathogens) due to human travel and the food 'business,' including traffic in live animals and associated products.

These different components of the human food chain are inextricably linked, and interactions between human, animal, plant, and environmental bacterial populations lead to increases in the incidence of antibiotic-resistant pathogens in various populations. This is a very serious matter and the threat of infection from an untreatable (resistant) food-borne pathogen has become a significant risk in daily life [56]. The implications for human health, especially among the more disease-susceptible sectors such as the very young and the elderly, must be addressed as a priority. For example, one of the first consequences of antibiotic usage in agriculture was the transfer of resistance genes from animal isolates (e.g., *S. typhimurium* 29) to human pathogens. In such instances, the animal strains, being essentially innocuous to humans, were simply providers of the resistance determinants. However, a growing number of multi-drug-resistant organisms which are pathogenic to both animals and humans, such as *S. typhimurium* DT104 and *Escherichia coli* O157:H7 [57–59], have been identified in animals, which is an entirely different problem and cause for concern. The incidence of food-borne illness is on the increase in all countries and there have been several outbreaks of bacterial disease (including a number of fatalities) as a result of the consumption of contaminated meat, dairy products, vegetables, and fruits; this demands immediate action on a worldwide scale [60].

Conclusions

It is apparent that although a general understanding of the origins, mechanisms of acquisition, and dissemination of antibiotic resistance genes within microbial populations exists at the moment, there are many missing links in this picture, largely because studies of antibiotic-resistant bacteria have focused on the properties of antibiotic-resistant pathogens isolated under human clinical situations and little effort has been expended in trying to establish how these strains were generated. For example, is it possible that any resistance determinant is accessible to any bacterial species within a given population? The effect of antibiotics on microbial populations in general, such as normal human or animal flora and soil ecosystems, must be explored to provide a rational basis for the institution of measures to control the problem.

Microbes have the genetic flexibility to develop resistance (or tolerance) to any antimicrobial agent; this applies to bacteria, fungi, protozoans, and viruses. Thus we must accept that the use of antimicrobial therapy is always going to be compromised. It is incumbent on scientists and the health care profession to adopt a flexible approach to this reality by developing a range of strategies based on epidemiology, diagnosis, and new antimicrobial agents. More intensive efforts in the discovery and development of novel antibiotics and effective vaccines are major priorities, but must be matched by improved technology applied in the agricultural and food supply industries to ensure that 'human' antibiotics are never employed for other purposes. The fact that most types of antibiotic-resistant microorganisms are firmly and ubiquitously ensconced presents a continuing primary treatment problem for the medical profession. Initiatives to reduce the development and incidence of antibiotic resistance in hospitals and the community must be introduced. For example, can less antibiotic be used more effectively? Can antibiotic use be limited to cases of demonstrated need and proven efficacy? Routine and effective monitoring of resistant organisms and tracking the movement of resistance genes through the operation of nationwide and international epidemiological networks is absolutely essential if resistance and resistant organisms are to be controlled.

The increasing concerns about emerging classes of pathogens and the development of untreatable strains have triggered an aggressive search for new antibiotics by the pharmaceutical industry. This will be aided substantially by increasing knowledge of the nucleotide sequences of the genomes of bacterial and fungal pathogens, thereby furnishing more detailed information on the genetic and biochemical mechanisms of microbial pathogenesis as well as identifying new metabolic targets for the design and discovery of novel classes of inhibitors. By the end of the year 2000, the complete nucleotide sequences of most of the major pathogens will have been completed. New types of chemical compounds are being generated in large numbers by combinatorial chemistry and these can be used to fuel the modern high-throughput screening programmes. In addition, the applications of a new methodology—combinatorial biology—has great promise in finding new types of microbially based drugs by providing access to the structural diversity of organisms that cannot be grown in the laboratory. Until now, the discovery of new antibiotics has been severely limited by the facts that (i) more than 99% of soil microbes cannot be grown and (ii) determination of the structures of com-

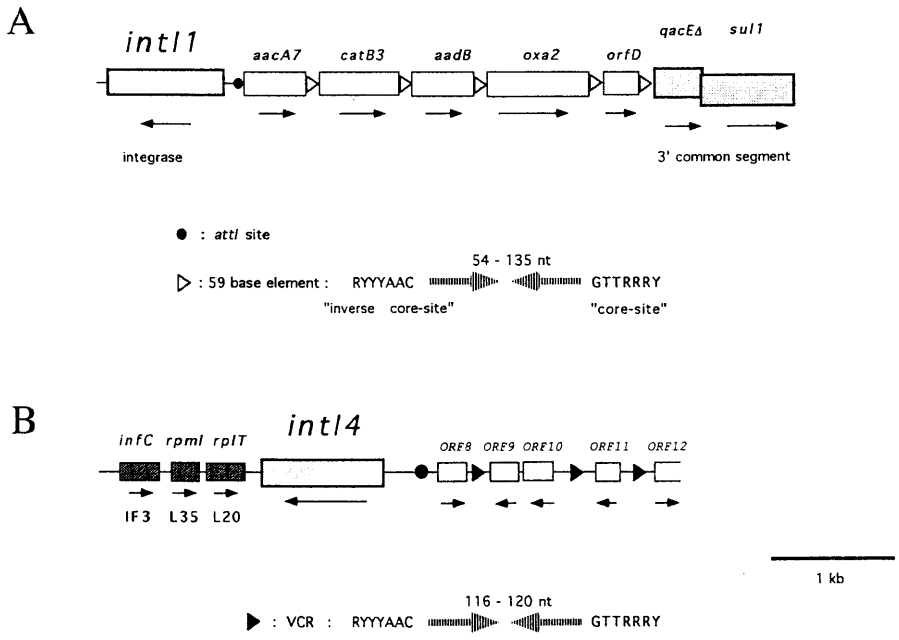

Figure 4. Comparison of the structures of 'classical' integrons (*A*) with those of *V. cholerae* repeats (VCRs) (*B*). (*A*) The various resistance genes are separated by '59-be elements' that have the consensus sequence shown. The 59-be elements are involved in integration at the *attI* site promoted by the integrase (intI). (*B*) The open reading frames (ORFs) are aligned downstream of a putative attachment site (●) and are separated by highly homologous VCRs for which the consensus sequences are shown. Up to 100 ORFs can be organized in such structures in *Vibrio* sp.

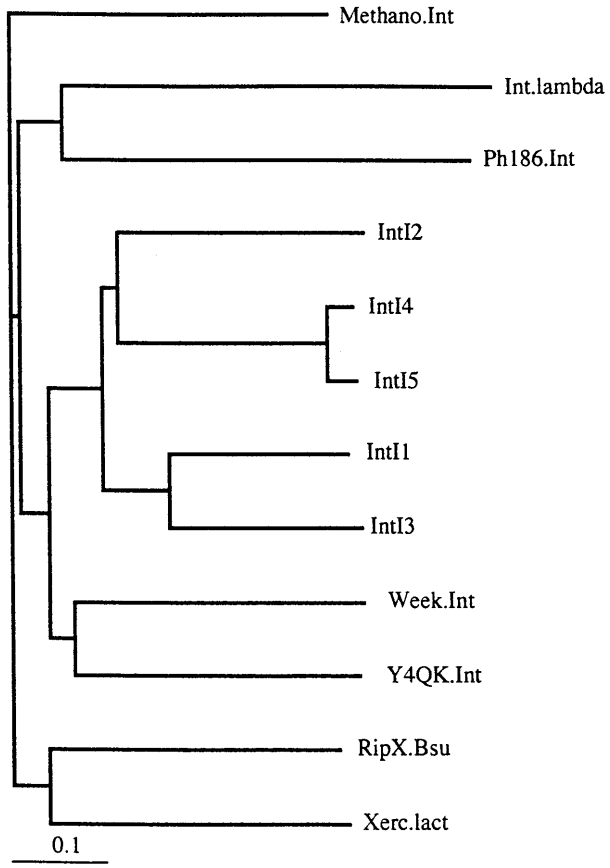

Figure 5. A dendogram showing the sequence relationships between various classes of integrase, indicating the clustering of integron and VCR integrases. Methano, *Methanobacterium thermoautotrophicum*; Week, *Weeksella zoohelcum*; Xerc.lact, *Lactobacillus leichmannii*, Y4QK, *Rhizobium* sp. NGR234 plasmid; Ph186, phage Phi 186; IntI1, class 1 integron; IntI2, class 2 integron; IntI3, class 3 integron; IntI4, *V. cholerae*; IntI5, *V. mimicus*; RipX, *Bacillus subtilis*; lambda, phage λ.

plex natural products (usually available in small quantities) is difficult. However, modern molecular methods permit the isolation of genes from non-cultivable microbes and their expression in surrogate hosts. With technical advances such as high-resolution mass spectroscopy and nuclear magnetic resonance analysis to provide more facile structure determination, natural products are once again coming into prominence in drug discovery programmes.

Is it possible to prevent the development of antibiotic resistance? Since the process of bacterial conjugation is likely to have been involved in the majority of the inter-specific and inter-generic traffic of antibiotic resistance genes in nature, it has been suggested that agents preventing such transfer would be valuable in limiting the spread of resistance. While this is an attractive notion, it is unlikely to be effective in practice. For one thing, there is more than one type of conjugation system (there are possibly more than 50!) genetically and mechanistically. Will it be feasible to target all types of conjugation-driven transfer with single agents? Second, while conjugation is probably the most effective process of horizontal gene transfer across a broad host range, it is not the only mechanism (for instance, transformation has been well defined for a number of genera and many different species can be so manipulated in the laboratory), and there are other processes of gene transfer in nature. It will be impossible to devise the means of blocking all of them. Interfering with gene transfer is a prophylactic process that may be applicable in limited and defined ecological niches but will not provide protection on a broader scale. Moreover, even if gene transfer is retarded, the antibiotic-resistant bacteria will remain in the population. Ultimately, the development of a range of safe and effective vaccines will permit the control, if not the eradication, of some infectious agents. As has been said previously, appropriate use of antimicrobial agents combined with the discovery of novel antibiotics is probably the most effective means of maintaining parity with infectious diseases.

Acknowledgements. We are grateful to Dorothy Davies for her diligent preparation of the manuscript. Dean Rowe-Magnus made useful comments on the text. We wish to thank the National Research Council of Canada, the Canadian Bacterial Diseases Network, and Institut Pasteur for generous support of this work.

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