Interactions among Strategies Associated with Bacterial Infection: Pathogenicity, Epidemicity, and Antibiotic Resistance†

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INTRODUCTION

The mechanisms involved in the virulence (defined as the relative capacity of a microbe to cause damage in a host [72, 73]) of pathogenic bacteria as well as those determining antibiotic resistance are important and widely studied topics in clinical microbiology. However, they have rarely been analyzed and integrated as we intend to do in the present review.

In terms of evolution and ecology, antibiotic resistance and virulence determinants share some basic characteristics. Since these determinants have been acquired by horizontal gene transfer from other organisms, many are examples of what has been termed "evolution in quantum leaps" (153). Also, most determinants serve to escape the action of antibacterial defense systems that have developed either by natural (host anti-infectious mechanisms) or cultural (antibiotics) evolution (123) to prevent infections. Both the natural anti-infective defenses and antibiotic treatments lead to stringent conditions for bacterial growth. In biology, any limiting condition for the majority is a golden opportunity for the minority. Those bacteria that are capable of surviving and multiplying under these conditions will gain access to organic spaces in which competition with other microorganisms is avoided (exclusive environments).

We might then assume that both virulence and antibiotic resistance are formally similar adaptive mechanisms selected to survive under stress conditions (either host invasion or antibiotic treatment). From an ecological point of view, both infective conditions and antibiotic treatments are evolutionary bottlenecks that tend to reduce microbial biodiversity, so that only a very specific subset of bacteria are capable of colonizing the host under those conditions (Fig. 1). There are several bacterial species that are able to grow at 37°C and are tolerant to the oxygen tension present in different parts of the human body. The fact that environmental microorganisms that are unable to produce disease in the healthy host frequently infect immunocompromised patients indicates that many organisms are ecologically compatible with the physicochemical conditions within the human body. The human body and its physicochemical conditions are then an ecological space that can be colonized by several microorganisms (182), frequently with an environmental origin (307). In the normal host, this potential for colonization is limited by the immune system, which actively impedes colonization of the human body by opportunistic pathogens. In the immunodepressed host, only antibiotic treatment maintains a small colonizable space in the human body (see below).

We want then to go one step beyond. Is there any evolutionary relationship between resistance and virulence? If modern medicine has limited the spread and maybe the evolution of bacterial pathogens, this has been done at the expense of increasing antibiotic resistance. Apparently, a decrease in the size of pathogenic populations and an increase in the number of antibiotic-resistant microorganisms (378) have characterized the evolution of infectious diseases. In pure theory, when the number of pathogens decrease to a critical value, antibiotics should be less required, and restoration of antibiotic susceptibility could be expected to occur. We know, however, that this is not true. The widespread dissemination of antibiotic resistance among bacterial populations (275) has maintained or even increased the number of harmful bacteria involved in infections. In fact, and in spite of previous claims, infectious diseases are among the most prominent health problem nowadays (419), in part as the consequence of the increasing number of antibiotic resistance phenotypes, which make bacterial infections untreatable by current therapeutic protocols (277, 329).

If new antibiotics and protocols are required for fighting infectious diseases, we must first understand the relationship between virulence, transmissibility, and antibiotic resistance. Thus, two essential topics are first reviewed in this work: the effect of acquisition of antibiotic resistance (and antibiotic treatment) on bacterial virulence, and analysis of whether pathogenicity and antibiotic resistance might prevail as linked phenotypes in the world of the future, if pathogens become antibiotic resistant as a consequence of intense antibiotic selective pressure.

GENETIC LINKAGE OF RESISTANCE AND VIRULENCE

Virulent bacteria have acquired their phenotype through a long evolutionary course in close contact with their natural hosts. Most virulence determinants are either located in chromosomal gene clusters (pathogenicity islands) or harbored in genetic accessory elements such as plasmids and phages. This suggests that evolution from an avirulent way of life to pathogenicity frequently implies the acquisition of foreign pieces of DNA (125, 153, 157). Nevertheless, for the organism to be a real pathogen, these pathogenicity factors should be inserted in an organism ecologically compatible with the potential host. Moreover, in some cases it is not an acquisition but a deletion (virulence-associated "black holes") which is needed to become a pathogen (254, 271, 359).
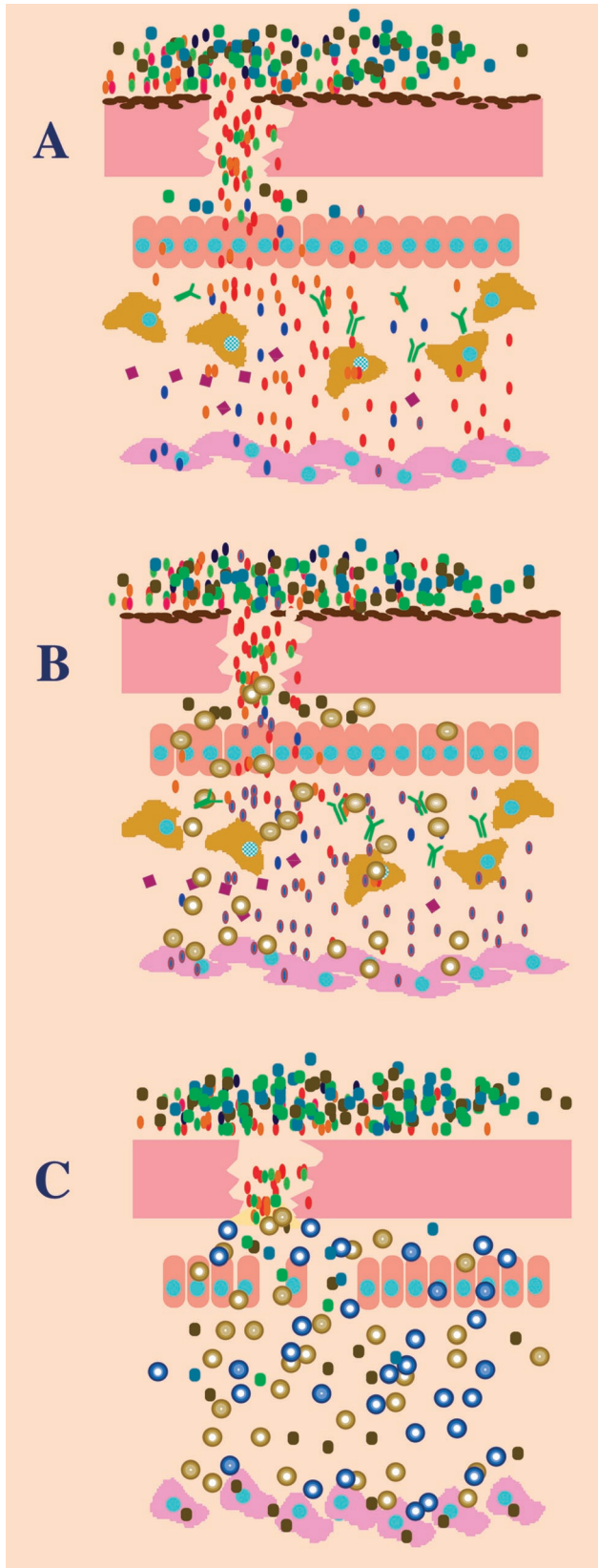
FIG. 1. Infection and antibiotic treatment are both stringent growing conditions. Several bacteria are able to grow at the temperature and oxygen tension of and using the nutrients present in the human body. However, only some are able to produce infection; this is shown

Indeed, any change in lifestyle has a biological cost, as functions needed in one habitat may cause a burden in another habitat and could therefore be counterselected. Acquisition of a virulence phenotype might then require the acquisition of some different pathogenicity islands and the loss of some chromosomal DNA regions. Therefore, the construction of a pathogen by the acquisition of specific pathogenic elements in ecologically compatible host-adapted bacterial genomes has probably occurred over a long evolutionary time.

In this regard, the record of infectious diseases (mainly epidemic ones) in human history and bioarchaeological analysis of the paleontological record indicate that we have been in contact with pathogens similar to those currently producing infections for a long time. In this way, it has been demonstrated that infectious diseases such as syphilis, tuberculosis, and other infections due to bacteria, viruses, and parasites were common in prehistoric men (420), particularly in the Neolithic age, when agriculture and farming ensured close contact between humans and between humans and animals. In some cases, however, such evolution has occurred in historical times, as the evolution from *Yersinia pseudotuberculosis* to *Y. pestis*, which was shortly followed by the first pandemic plagues (2). Obviously, the evolutionary outcome of pathogenic organisms tends to be limited in modern times by the anti-infectious repertoire developed by humans that includes hygienic measures, epidemiological controls, vaccines, and, significantly, antibiotics.

Conversely, acquisition and further spread of antibiotic resistance genes among pathogenic bacteria is a phenomenon that has occurred just in the last 50 years as a consequence of extensive antibiotic use for human therapy and animal farming. At first glance, pathogenicity and resistance should be unlinked phenomena. However, several examples indicate that this is not the situation for several bacterial pathogens. Antibiotic resistance and virulence genes can be linked (and then co-

in panel A. Several bacterial species (ovals are pathogens, circles are environmental ones) coexist outside the host. Some species are able to displace the commensal flora, traverse through different epithelia, resist the action of macrophages, antibodies, defensins (with squares), and all the anti-infectious mechanisms of the human body, to finally reach the target cells where the disease is produced. At any of these sequential bottlenecks, only some bacteria are selected, and their population is further amplified, so that, from the high variability of bacteria that could potentially produce the disease, only some are really pathogenic. In panel B, the same situation is analyzed but under antibiotic treatment (spheres). In this case, only a small proportion of the cells belonging to the infectious species (the antibiotic-resistant ones; blue ovals with a red line) are able to produce infection, so that the antibiotic-treated host is a more stringent ecosystem for the growth of bacteria. However, in the case of a debilitated person (panel C), the situation is somewhat different. In these patients, the indigenous microflora might be removed because of antibiotic use, the epithelial integrity may be impaired (intubated patients), the immune system can be abolished (immunocompromised patients), and even the target cells can change. Under these circumstances, some environmental species can produce infection (opportunistic pathogens), because growth conditions are less stringent. However, at least in the developed world, those patients are usually under heavy antibiotic treatment, frequently with a combination of antibiotics (golden and blue spheres), so that only those species with an intrinsically antibiotic-resistant phenotype can produce the infection. Under these circumstances, the main selective force is antibiotics, so that antibiotic treatment becomes a risk factor for some pathogens.

selected) in the same replicon, or eventually a single determinant can be involved in both virulence and resistance. Some examples of these situations will be reviewed.

Common Mechanisms Involved in Virulence and Resistance

In this section, we analyze whether a mechanism which has been selected on the basis of a bacterial virulence phenotype might also be relevant for antibiotic resistance and vice versa.

Let us first examine the effect of the spatial localization of bacteria in the human body. Several virulent bacteria base their pathogenic characteristics in an intracellular way of life during infection (125). Internalization may be required for the induction of inflammatory cytokines (166) and produce tissue damage through the induction of either necrotic (130) or apoptotic (167, 410) responses. A remarkable example is the apoptosis of macrophages induced by pathogens such as *Shigella*, which both avoid the antibacterial effect of those cells and trigger inflammation (427, 428). In some well-characterized examples, bacteria are able to travel from cell to cell without any significant contact with the extracellular milieu (43, 273), thus avoiding the immune system as well as contact with antibiotics that do not enter mammalian cells (12, 172). This provides the first clue as to how well-characterized virulence phenotypes can be related to antibiotic resistance.

Mammalian cells are poorly permeable to or easily exclude several families of antibiotics (12, 166). Even if the antibiotic enters the mammalian cells, intracellular growth might induce a transient antibiotic-resistant phenotype, a situation that has been described for *Legionella pneumophila* (35). The location within the host may alter in an unspecific way the susceptibility of the bacterial organism to antibiotics. *Haemophilus influenzae* grown in animals undergoes modifications in penicillin-binding proteins, as peptidoglycan metabolism is directly affected by the environment (93), and *Salmonella* peptidoglycan is dramatically altered for bacteria growing intracellularly (308). Although the effect of these changes on antibiotic susceptibility has not been analyzed extensively, it is possible that those metabolic changes may alter the activity of antibiotics such as beta-lactams against bacteria growing during infection. It is then possible that intracellular localization may allow bacteria to maintain a phenotype of antibiotic resistance.

In this respect, treatment with some antibiotics might select for intracellular clones. This could be the case for species in which some clones are able to invade mammalian cells and others are noninvasive; one such species is *Pseudomonas aeruginosa* (126). On the contrary, some antibiotics, such as macrolides and fluoroquinolones, are actively accumulated by human cells (262). In this case, intracellular bacterial localization might eventually increase the antibiotic effect against bacterial pathogens, leading to better eradication and favoring the less invasive strains.

A similar situation of phenotypic antibiotic resistance can be observed for bacteria growing in biofilms. Bacterial biofilms are frequent in persistent infections (85), such as those associated with cystic fibrosis (354), chronic bronchitis, osteomyelitis (152), and foreign-body-associated infections. Bacteria growing in biofilms are more resistant than those under a planktonic way of life to the action of phagocytic cells' antibacterial activity (261) as well as the action of antibiotics (16,

83, 149, 370, 404). As in the previous case, antibiotics might select biofilm-forming organisms, thereby increasing the prevalence of chronic infections. Other mechanisms of virulence could prevent the action of antibiotics against bacteria. For instance, toxins leading to local necrosis or abscess formation certainly will decrease the local availability of antibiotics due to reduction in antibiotic arrival to the foci or because of local inactivation of the drugs by altered pH, free proteins, or DNA. In these cases, the mechanisms of pathogenicity may finally serve as mechanisms for antibiotic resistance.

A final example of a virulence mechanism with a role in antibiotic resistance is presented by *Bordetella pertussis*, the etiological agent of whooping cough. The cell wall of the virulent strains of this microorganism is infrequently susceptible to autolysis triggered by beta-lactam antibiotics; only avirulent *B. pertussis* strains are known to be lysed. It was demonstrated that this phenotypic tolerance and the antibiotic-induced autolytic activity are controlled by the *vir* locus, which determines phase transition in *B. pertussis*, a key element in the virulence properties of this bacterial species (389).

In previous examples, we have seen that a "virulent way of life" can contribute to an antibiotic resistance phenotype. Could antibiotic resistance determinants also contribute to bacterial virulence? Some examples suggest that this could be the case. Recently, one area of intense study of antibiotic resistance is the analysis of multidrug resistance (MDR) efflux pumps (280, 293). These determinants are able to extrude an ample range of substances that include antibiotics, solvents, dyes, and quorum-sensing signals (295, 423). Extrusion by the same pump of compounds as diverse as erythromycin, quinolones, beta-lactams, and ethidium bromide is a rule more than an exception.

Might these MDR determinants extrude compounds involved in the host defense be contributing to bacterial virulence? The answer is yes. For instance, adaptation to growth in the presence of bile salts is a prerequisite for any pathogen to colonize the intestinal tract. It has been reported that *Escherichia coli* extrudes bile salts (382) through the *acrAB* system, which was first characterized as an MDR determinant (6, 230); the same occurs in *Salmonella enterica* serotype Typhimurium. In fact, it has been suggested that extrusion of bile salts present in the intestinal ecosystem to which these bacteria are adapted is the function for which those MDR determinants were selected (230). Analysis of *S. enterica* serotype Typhimurium mutants with high susceptibility to bile salts has demonstrated that this higher susceptibility was a consequence of inactivation of the MDR determinant *acrB*. This inactivation leads to a reduced ability to colonize the intestinal tract in a murine infection model (199), which indicates that *acrAB* is involved in both antibiotic resistance and virulence.

Some MDR systems, such as Mtr from *Neisseria gonorrhoeae* (158) and SapA (291) from *S. enterica* serotype Typhimurium, can actively extrude both defensins and antibiotics, thus contributing to reducing antibiotic susceptibility as well as to increasing the number of virulence determinants of the organisms. In fact, it has been described that resistance to defensins is required for a full virulence phenotype in the case of *Salmonella* (154). The mechanisms of action of defensins resemble at least in part that of peptidic antibiotics currently used in clinical practice (180, 391). Thus, it is conceivable that they

might share the same mechanisms for resistance. This situation has been described for polymyxin B. The sensitivities to polycationic peptides were compared between two groups of *Yersinia enterocolitica* isolates, one with an environmental origin and the other from infections (38). Data indicated that pathogenic strains of *Y. enterocolitica* were resistant to defensins and also to polymyxin B, showing a clear cross-linkage between antibiotic resistance and virulence as a consequence of the expression of a resistance determinant.

Not only MDR pumps but also biocide efflux determinants may play a role in bacterial virulence. It has been reported that the QacA pump of *Staphylococcus aureus*, involved in resistance to several organic cations (biocides included), has a role in resistance to thrombin-induced platelet microbicidal protein (197). Thus, the presence of the pump contributes to the survival of strains carrying this determinant at sites of endothelial damage as well as in experimental endovascular infections. We want to stress here that the mechanisms of extrusion of antibiotics are similar to those translocating some proteins involved in pathogenicity. The hemolysin export apparatus in *Escherichia coli* comprises the outer membrane channel trimmer protein TolC that is also involved in the AcrA-B multidrug efflux transport complex (279). The periplasmic proteins of both systems, AcrA and HlyD, have a similar structure and are able to interact with TolC (278).

In all previously discussed examples, a mechanism selected for bacterial virulence can also produce an antibiotic resistance phenotype. Selection for the most virulent strains might select for antibiotic resistance, and conversely, selection for antibiotic resistance might select the most virulent microorganisms. However, the opposite situation can also be found: antibiotic resistance may decrease bacterial virulence (see below). For instance, the KatG catalase-peroxidase activity is important for the survival of *Mycobacterium tuberculosis* in the host (216). Mutations that eliminate this activity prevent the activation of isoniazid and are the major cause of resistance to this drug in *Mycobacterium* spp. (316, 425). It might then be predicted that isoniazid-resistant *Mycobacterium* mutants might be less virulent than wild-type isoniazid-susceptible strains, at least in the case of *M. tuberculosis* (264) and *Mycobacterium bovis* (414). However, when isoniazid-resistant isolates were analyzed, such reduced virulence was not found. It turned out that *M. tuberculosis* pathogenic isolates accumulate compensatory mutations in the gene encoding the alkyl peroxidase AphC, which can substitute for KatG for survival inside the host (350).

Costs of and Compensations for Virulence and Resistance

It is largely assumed that acquisition of novel genetic determinants may have a cost for the bacterial host. That may happen because of partial incompatibility of previous and acquired lifestyles, or because of the extra energy required to maintain the genetic vectors carrying the new genes. The acquisition of plasmids or antibiotic resistance-virulence genes might have an effect on bacterial fitness (8, 13, 426). As previously stated, pathogenicity islands, acquired through horizontal transfer of large gene arrangements, have a major role in bacterial virulence (157). Also, large *vir* plasmids are needed for some pathogens, such as *Shigella* (337, 406) and *Yersinia* (306), to express a pathogenic phenotype.

We might speculate that acquisition of these determinants implied a cost in bacterial fitness when they were included in the genome of the previously nonvirulent bacteria. However, acquisition of resistance genes by pathogenic bacteria has occurred over the last 50 years, but acquisition and further evolution of structures as pathogenicity islands occurred thousands of years ago. Thus, the evolutionary time needed to acquire and optimize compensatory mutations to alleviate the effect on fitness of the acquisition of pathogenicity determinants has been much longer. Also, for plasmids carrying *vir* determinants, the costs of the acquisition of such determinants might be compensated for by mutations in other loci. Of note, it has been shown that the *vir* plasmid can be easily lost in vitro in *Shigella* (342). However, growth conditions in vivo should preserve the plasmid's presence during infection, otherwise *Shigella* would not be virulent anymore.

Acquisition of *vir* plasmids ensures the ability to colonize a different biological compartment, thus evading competition with other bacterial species and eventually reducing the potential fitness cost imposed by the new genetic element. The same has probably occurred for enteroinvasive *E. coli* isolates (335, 336). Recent work suggests that the invasive ability of those isolates has evolved in different chromosomal backgrounds, probably through the spread of plasmid-borne invasion genes, and the maintenance of invasive phenotypes in separate lineages suggests that this ability confers a selective advantage to invasive strains (247). Although the Ewald hypothesis (119, 120) is still controversial, it can be speculated that sanitation procedures, vaccination, and widespread antibiotic use, impeding host-to-host spread of *vir* plasmid-based pathogens, may reduce the overall pathogenic power of microorganisms.

Due to the rapid acquisition and fixation of compensatory mutations by bacteria, experiments to evaluate the biological cost of the expression of novel antibiotic resistance and virulence determinants are difficult to perform. Antibiotic resistance and virulence can be acquired either by horizontal transfer of antibiotic resistance genes (97) or by mutation (241). The presence of new plasmids and transposons in the bacterial genome has a relevant cost for the recipient bacteria (13). However, it has been demonstrated that under these circumstances, the cost is compensated for in few generations as the consequence of genetic change by the host, getting the specific plasmid-bacterial strain association more fit than the previous non-plasmid-containing bacterium (61).

Also, in the case of mutations leading to antibiotic resistance, some examples demonstrate the possibility of fitness reduction, which may also reduce bacterial virulence (see last example in previous section). For instance, current evidence supports the idea that highly fluoroquinolone-resistant *Salmonella* strains could be counterselected in the field (145). A substantial reduction of the virulent characteristics has been also described for antibiotic-resistant *S. enterica* serotype Typhimurium isolates (48). Although this may occur for some bacteria, it does not mean that it will always occur. In fact, an outbreak of quinolone-resistant *S. enterica* serotype Typhimurium DT104 has recently been described (268), and an increase in quinolone-resistant *Campylobacter jejuni* infections has been reported in Minnesota (356).

The emergence of compensatory mutations rapidly alleviates the biological cost of antibiotic resistance in *S. enterica*

serotype Typhimurium isolates (45), and this could be the situation for the aforementioned outbreaks of quinolone-resistant bacteria. Noteworthy, the compensatory mutations that are selected in vivo and in vitro are different, reflecting the different growing conditions for bacteria during infection compared with microorganisms growing in vitro (49). This indicates that the metabolic requirements needed for infection are different from those for surviving in the environment. Therefore, acquisition of the characteristics required for infection and antibiotic resistance might make them less proficient for surviving in the environment and to be transmitted among different hosts.

It seems then that acquisition of novel virulence and antibiotic resistance traits has a cost in bacterial fitness, but the cost is rapidly compensated for due to the high plasticity of bacterial genomes and the huge populations of bacteria from which compensatory mutants can be selected (59, 209, 213). However, this situation may not always occur. Possibly, only those mechanisms that can be compensated for by mutations in other loci are selected because these are the only ones that can avoid fitness reduction. As stated by other authors (13), the effect of antibiotic resistance (and in a higher grade of virulence determinants) on bacterial fitness has been properly addressed in only a few studies. This topic needs to be investigated so that the biological potential of novel virulent and antibiotic-resistant bacteria may be predicted.

Evolution and Dissemination of Genes Involved in Virulence and Resistance

As previously stated, both antibiotic resistance genes and virulence determinants have been acquired by horizontal gene transfer in most cases (285). One intriguing question that has not been completely resolved resides in the origin of these determinants.

It is widely accepted by the plasmids and transposons that carry antibiotic resistance genes may have originated in antibiotic-producing organisms in order to avoid the deleterious effect of the antibiotic on themselves (39, 98, 239, 290, 407). Eventually, they could have further evolved in organisms in an ecological consortium with antibiotic producers. More recent works indicate that this hypothesis about the origin of resistance is only half true (11, 98). For instance, it is difficult to believe that chromosomal beta-lactamases (71, 217) as well as some aminoglycoside-inactivating enzymes (4, 201, 233) and a plethora of MDR determinants (280, 293) which are present in all isolates of a given bacterial species originated in the antibiotic producers. In fact, all these determinants must have a functional role other than antibiotic resistance, and this phenotype will only be a consequence of their primary physiological function (see reference 11 for a review of this concept).

In the case of virulence determinants, the current paradigm indicates that pathogenicity islands acquired through horizontal gene transfer are frequently responsible for the pathogenic properties of virulent bacterial species (125, 153, 157). Different from antibiotic resistance genes, the pathogenicity islands are more difficult to explain, because pathogenic bacterial ancestors carrying such gene clusters have not been detected. It has been proposed that pathogenicity islands might be relevant for the biodegradative properties of microorganisms (including

decomposition of dead bodies), to kill living cells (to obtain food and reduce competition), and to live inside eukaryotic cells (such as amoeba, protozoa, plants, and animals) in natural environments (161).

Antibiotic resistance genes and virulence determinants might then play a different role in the original organisms from which they were transferred to pathogenic bacteria. However, once those determinants have been selected in the heterologous host, they confer a selective advantage and are fixed in the pathogenic bacterial populations, where they can evolve further and eventually be transferred to other bacterial species.

Mutation and recombination. Two different processes account for evolution of antibiotic resistance-virulence phenotypes: gene mutation and gene recombination (horizontal gene transfer included). The role of mutation in antibiotic resistance is well known (241). In the case of virulence, fewer examples of such a role have been described. However, mutational activation-inactivation of intrinsic genes might also contribute to a virulent phenotype in the case of opportunistic pathogens. As an example of cryptic virulence determinants activatable by mutations, laboratory isolates of *E. coli* are hemolytic (105) because of mutations in the *hns* (147) and *fnr* (315) genes. Whether this is just a laboratory curiosity and might actually have a role in the course of nosocomial infections by *E. coli* is a matter of speculation.

Some virulence determinants of *P. aeruginosa*, such as alginate production (60) and cytotoxicity, are downregulated. However, mutants in which the expression of such determinants is derepressed are frequently found during infection (240). This indicates that mutation probably plays a major role in the emergence of the different virulent phenotypes shown by *P. aeruginosa* clinical isolates. Pathogenic *P. aeruginosa* isolates can be broadly classified into two groups, those with a cytoinvasive phenotype and those with a cytotoxic phenotype. However, both kinds of isolates contain the genes required for invading epithelial cells. Invasive and cytotoxic strains differ in the expression of the genes under the control of an activator called ExsA (116). Mutations in the gene *exsA*, which encodes the activator, lead to not a cytotoxic but only an invasive phenotype (116, 126). Although both types of bacteria are virulent, they occupy different environments (inside and outside epithelial cells), so that one or the other phenotype might be selected in vivo by, for the moment, unknown processes.

Recombination also has an important role in the evolution of antibiotic resistance determinants. Progressive clustering of genes, leading to an operon structure, may have occurred to optimize and regulate the expression of ancient genes producing a resistance (frequently low-level resistance) phenotype (206). In a similar way, the construction of a pathogenicity island requires the recruitment of different genes that recombine to produce a single genetic element containing several genes with a common role, virulence. In other cases, intragenic recombination is essential to produce a resistant phenotype. The most conspicuous example of recombination in chromosomal DNA leading to antibiotic resistance is beta-lactam resistance produced by recombination of penicillin-binding-protein genes in *Neisseria gonorrhoeae* and *Streptococcus pneumoniae* (79, 235).

Similar recombinatorial processes may have influenced the evolution of virulence. However, unlike for antibiotic resis-

tance, few examples of recent evolution of bacteria to a virulent phenotype have been described. One might be recombination between different sets of genes, which causes the rearrangements that can be observed in the capsular antigens of *S. pneumoniae* (78–80, 276) and can be considered a mechanism of defense against host immunity. Another could be the emergence and further dissemination of enterohemorrhagic *E. coli* O157:H7 strains (131). This *E. coli* serogroup has emerged as a relevant pathogen in the last 20 years. Enterohemorrhagic *E. coli* isolates contain virulence plasmids and pathogenicity islands similar to those found in *Shigella* spp. (70, 237). Although it was a matter of speculation whether these genetic determinants were acquired recently by *E. coli*, the recent sequencing of the genome of an *E. coli* O157:H7 strain (297) demonstrated that it contains as many as 1,387 new genes in comparison with the previously sequenced nonpathogenic laboratory strain *E. coli* K-12 (52).

We must be aware that acquisition of pathogenicity determinants by previously nonpathogenic organisms might occur in a similar way as it happens with antibiotic resistance determinants. For instance, the acquisition of virulence plasmids by bacteria forming part of the human indigenous flora and thus already well adapted for surviving inside their host (such as *E. coli*) might lead to the emergence of novel pathogens. In a similar way, the acquisition of virulence determinants by environmental microorganisms should produce novel pathogenic bacteria. Although the evolution of novel pathogens is possible, the selective pressure in favor of the selection of virulence determinants is not as strong as in the case of antibiotic resistance determinants. Therefore, an explosion of novel pathogens, such as has occurred with antibiotic-resistant bacteria, will most likely not happen in the near future, not only because the selective pressure for a pathogenic phenotype is less strong than for an antibiotic resistance phenotype, but because selection for pathogenicity in today's world might decrease with sanitation (119, 120) if the Ewald hypothesis is true.

A critical point to discuss here is that virulence and/or antibiotic treatment might increase the rate of bacterial variation. In the case of infection, this situation might contribute to increased mutation rates (310, 326) and even to the selection of hypermutator strains (see below), thus enhancing antibiotic resistance mutation (67, 208, 287, 380). In a similar way, antibiotic challenge might also produce hypermutable phenotypes (9, 318, 320), increasing the possibility of mutants overexpressing virulence determinants. Mechanisms favoring hypermutation may also facilitate recombination (253). Moreover, in vivo transfer of plasmids carrying antibiotic resistance genes and/or virulence determinants and recombination are probably favored during infection due to host signals that enhance gene transfer (260).

In this respect, the effect of antibiotics in inducing the transfer of plasmid and transposons has been demonstrated in vitro (387). It has been described that the same genes required to initiate infection of human macrophages by *Legionella pneumophila* are involved in plasmid mobilization (344). Recent results in our laboratory also suggest that bacterial expression of factors involved in cell-to-cell DNA transfer (likely antibiotic resistance plasmids) in some organisms, such as *S. pneumoniae*, may be triggered by inflammatory products (M. R. Baquero, unpublished results). It can then be suggested that

TABLE 1. Examples of virulence determinants encoded by antibiotic resistance plasmids

Determinant	Reference(s)
Heat-stable enterotoxin	132
Heat-labile enterotoxin	132
Aerobactin	168, 403
Serum resistance	168
Hemolysin	352
Microcin D93	245
Colicins	132
Bacteriocin	272
Vibrio cholerae cytotoxin	36
vir plasmid pZM3	81
vir plasmid Dt66	96
vir plasmid pTE195	333
Cell invasiveness	339

bacteria are expected to evolve more rapidly inside the host and under antibiotic selective pressure, so that an infected patient under antibiotic therapy may act as an evolutionary accelerator.

Plasmids. It is well known that plasmids are major vectors for the dissemination of both antibiotic resistance and virulence determinants among bacterial populations. It is also clear that the presence of virulence and antibiotic resistance determinants in the same genetic element will produce coselection of both types of determinants. This applies as well for genes present in transposons, phages, and cassettes, which are discussed below. For bacteria carrying those linked determinants, the selection of an infective population will select for antibiotic resistance, and antibiotic selective pressure will select the virulence trait. Table 1 shows some published examples regarding this type of gene linkage. Among them, antibiotic resistance plasmids carrying, alone and in combination, genes encoding the synthesis of bacteriocins (272), siderophores (82, 168, 401), cytotoxins (34, 132), and adhesion factors (202) have been described.

General plasmid-encoded transfer functions (present in all transferable resistance plasmids) may be relevant by themselves for bacterial virulence. One of these functions is related to the *traT* genes, involved in plasmid conjugation (374). The other function is the production of pheromones (77), also involved in conjugation of plasmids and transposons in grampositive bacteria. Conjugative plasmids have a relevant role in the dissemination of antibiotic resistance. Plasmid conjugation is encoded in the *tra* region. It has been demonstrated that expression of *traT* genes might be involved in bacterial resistance to serum (181, 270), biofilm development (141), and phagocytosis (3), therefore contributing to the virulent properties of bacteria carrying conjugative (frequently antibiotic resistance) plasmids.

The plasmid transfer proteins are sometimes chromosomally encoded, but they can have a role in both plasmid transfer and virulence. This could be the case for *Legionella pneumophila*. It has been described that the *dot* virulence genes encode a large putative membrane complex that functions as a secretion system that is able to transfer plasmid DNA from one cell to another (402). Mutations in the *dot* genes reduced both virulence and plasmid transfer. In the case of gram-positive bacteria, aggregation substances encoded by pheromone plasmids

are involved in the clumping required for an efficient transfer of DNA by conjugation. It has also been described that *Enterococcus faecalis* aggregation substance promotes resistance to killing by human neutrophils in spite of phagocytosis and neutrophil activation (314) and enhances pathogenicity in a rabbit model (340) and thus, as said by R. Wirth, is "more than a plasmid collection mechanism" (416).

The analysis of plasmids from the preantibiotic era demonstrated that there have not been major changes in the families of plasmids present in pathogenic bacterial populations, but just recruitment of antibiotic resistance genes by formerly "antibiotic-susceptible" plasmids (95, 171, 179). Since plasmids encoding adaptive traits (eventually involved in the pathogenic lifestyle) certainly preceded antibiotic resistance, it could be suspected that many current resistance plasmids contain genes with a role in bacterial colonization and virulence. In fact, this has probably been the origin of plasmids containing virulence determinants and antibiotic resistance genes such as those listed in Table 1. We do not know, however, if this association is the rule for antibiotic resistance plasmids, because only a very small fraction of these determinants have been entirely sequenced.

Transposons. Transposons are also relevant for the dissemination of antibiotic resistance genes, either by integration in transferable plasmids or by direct conjugation and further integration in the bacterial chromosome. Also, transposons containing virulence determinants have been described. One example is the aerobactin operon (46, 142, 244). Aerobactin is a siderophore produced by several bacterial species (74). It has been proposed that aerobactin is a virulence factor with relevance for iron acquisition at the site of infection (103). Aerobactin genes have been found in the chromosomes of different bacterial species and in several different plasmids, a situation which indicates easy mobilization. The aerobactin operon forms part of a transposable element (102), and it has been proposed that it is part of a pathogenicity island in *Shigella flexneri* (403). Other virulence determinants found in transposable elements are the *E. coli* enterotoxin STII gene (170), the Shiga toxin operon, which has been found in a putative composite transposon in *Shigella dysenteriae* 1 (257), and the toxic shock toxin, carried by a family of mobile pathogenicity islands in *Staphylococcus aureus* (218).

Little is known about the association between antibiotic resistance and virulence determinants in the same transposon. However, there is no reason for this lack of association. Indeed, like other complex gene arrangements (see below), transposons frequently have a mosaic structure in which highly recombinogenic regions are included (56, 90, 205), so that the acquisition of novel traits is a common occurrence in transposon evolution (256).

Phages. The presence of virulence determinants in phages infecting different bacterial species has been described (Table 2). Also, a phage origin has been proposed for at least some pathogenicity islands (see below). Bacteriophage-associated transduction of antibiotic resistance determinants has been described as well (50, 51, 341, 390). Nevertheless, the presence of antibiotic resistance genes together with virulence determinants in the same phage has not been reported. A possible explanation for this phenomenon is the size requirements for phage DNAs. Phage DNA needs to accommodate inside the

TABLE 2. Examples of virulence determinants encoded by phages

Determinant	Reference(s)
Type III effector protein SopE	267
Shiga toxin	304
Adhesion factor	36, 400
Pathogenicity island and type IV pilus	184
Cholera toxin	121, 405
Survival in Peyer's patches	366
Exotoxin A from *Streptococcus pyogenes*	408

head of the phage particle, so that its length must fit a fixed range of sizes. In such circumstances, the gain of some genes must be accompanied by the loss of others. Because of this, the possibility of combining antibiotic resistance genes and virulence determinants in the same bacteriophage is lower than in the case of plasmids, which have less stringent requirements for the incorporation of novel DNA fragments.

Gene cassettes. In the last few years, several works have shown the presence of gene cassettes capable of integrating novel genes from different DNA sources, offering possibilities for the horizontal dissemination-linked antibiotic resistance-virulence genes. The most relevant family of these gene cassettes is the integron family (328, 377). Integrons are the primary systems for the capture of antibiotic resistance genes in gram-negative bacteria and have also been described in gram-positive bacteria. Integrons are formed by gene cassettes located downstream of a recombinase-encoding conserved sequence that includes a strong promoter (Fig. 2). This organization allows the formation of large arrays of gene cassettes which can eventually be transferred as a whole between different replicons.

Most of the integrons described to date are formed by antibiotic resistance genes (328). However, several different genes, including not only antibiotic resistance but also virulence determinants, have been found in integrons. Prototypic of composite integrons containing virulence genes are the VCR cassettes found in the chromosome of *Vibrio cholerae*. VCRs (from *Vibrio cholerae* repeated sequences) are a family of 123- to 126-bp sequences of imperfect dyad symmetry, highly repeated in the chromosome of *V. cholerae* (up to 60 copies). Analysis of VCR clusters demonstrated that the gene-VCR organization is identical to that of the antibiotic resistance cassettes present in integrons (255). Also, the formation
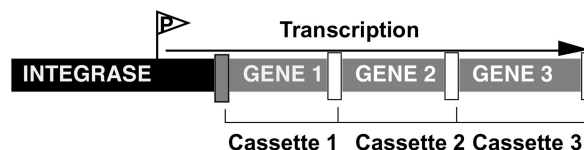
FIG. 2. Structure of an integron. Integrons are site-specific recombination elements that mediate the acquisition and spread of genes among bacterial populations. Integrons are formed by an integrase gene followed by one primary *att* recombination site (dark grey box) and several cassettes, each including one gene and one 59-bp recombination site (white square). The transcription of the system is controlled by a strong promoter located upstream of the integron. This structure favors the arrangement of genes in tandem, which are transferred as single elements among bacterial populations.

of the VCR islands likely occurred by means of an integrase-mediated process. This indicates that VCR islands might have a role in gene capture similar to the proposed role of integrons in the acquisition of antibiotic resistance genes. It cannot be dismissed that some genes in VCRs may play a role in antibiotic resistance, perhaps to still undiscovered antibiotics.

Another type of gene cassette with a key role in the establishment of the infective process are pathogenicity islands. These DNA regions found in the chromosomes and plasmids of bacterial pathogens are composed of clusters of genes (typically from 15 to 25, although more are possible) which have been acquired by horizontal gene transfer (157). It has been shown that several of these gene arrangements have been introduced into tRNA genes through a phage-mediated transfer mechanism (75). However, a role for insertion sequences has also been suggested in other cases. This is the case for the pathogenicity island required for growth of *Y. pestis* in iron-deprived environments (162). This island is flanked by direct repeats of IS*100*, an insertion sequence which is present in the chromosome and plasmid of this bacterial species and thus may contribute to recombination of genes from different replicons.

The presence of sequences with a role in DNA mobility either inside or flanking pathogenicity islands is quite common (157). However, few of these gene clusters have been demonstrated to be mobile. One example is the aforementioned family of pathogenicity islands carrying the gene encoding toxic shock toxin in *Staphylococcus aureus* (218). Stabilization of pathogenicity islands in the bacterial genome probably requires inactivation of the functions involved in mobility. In fact, the "mobility elements" present in pathogenicity islands frequently carry a large number of mutations, often leading to stop codons (21), which abolish the expression of such functions. Antibiotic resistance determinants have not been detected in pathogenicity islands, but that is an open possibility.

The transfer of gene clusters (contained in either plasmids, transposons, bacteriophages, or gene cassettes) is extremely relevant to bacterial adaptation to novel environments because it allows "bacterial evolution in quantum leaps" (153). As previously stated, both bacterial virulence and antibiotic resistance can be considered strategies to explore and colonize novel environments in which bacterial competitors are scarce. Bacteria will make use of all the tools (mutation, gene transfer, recombination) which allow the high plasticity shown by bacterial genomes (176, 211, 285, 349). In the treated host, adaptation of pathogens will require both antibiotic resistance elements and virulence determinants, so that both adaptive mechanisms must evolve together to produce the explosive spread of virulent and antibiotic-resistant microorganisms that we are now facing.

Phenotypic Adaptation in Virulence and Antibiotic Resistance

Over the course of an infection, bacteria must face several different environments (Fig. 1), so that phenotypic adaptation to all of them is an important trait for the final pathogenicity result. In this respect, both virulence and antibiotic resistance determinants can be environmentally regulated. The signaling network that regulates the expression of bacterial virulence

and antibiotic resistance determinants during infection is a hot topic in current research in microbial pathogenesis.

Environmental signaling in virulence and antibiotic resistance. Bacteria are constantly sensing the environment in order to respond to changes in its composition. In the case of bacterial pathogens, constant cross talk occurs between the microorganisms and their hosts (86) which modulates the expression of several genes in both the bacterial and host cells. Physiological signals such as calcium concentration (372), low pH (128, 296), high temperature (194, 296), and low iron concentration (88, 399) among others, trigger the expression of several virulence genes. However, little is known about whether such signals might also trigger the expression of antibiotic resistance genes.

Nevertheless, we know that the antibiotic susceptibility of bacteria is modulated by several factors which include growth phase (164), pH (89, 203, 226), carbon dioxide (226), temperature (311, 312), bile salts (230), and low iron (305). It is then clear that certain conditions during infection might induce the expression of both antibiotic resistance and virulence genes, and a common regulation of both types of determinants might then occur. Most probably this regulation does not always involve the same regulatory network, but in some cases virulence and antibiotic resistance genes can be part of the same regulon. In this regard, it has recently been described that the MarA protein (from multiple antibiotic resistance) of *E. coli* regulates the expression of more than 60 chromosomal genes. These genes include not only MDR determinants (156), but also genes with a potential role in virulence, such as those involved in oxidative stress and iron metabolism (33).

In general, a linkage between antibiotic resistance and virulence gene regulation might occur in global regulons, which modulates the expression of stress genes (see below). This type of regulation, coupled with phenotypic resistance of bacteria growing in biofilms, inside cells, and even in resting cells which are resistant to antibiotics, might produce situations of in host resistance at the site of infection that is impossible to predict by routine laboratory susceptibility tests (reviewed in reference 242).

Antibiotics as effectors of bacterial virulence. Common models of bacterial virulence analyze host-pathogen interactions under a defined set of conditions that usually do not include antibiotic therapy. Although the first stages of an infection usually occur without treatment, once a diagnosis is available, most infectious diseases evolve under antibiotic treatment. We want to stress here that antibiotics are not only bacterial killers, but also modulators of bacterial and even host cell transcription of different genes (238, 347). In such a way, antibiotics modulate the interaction between host cells and bacteria (289). It has also been suggested that they may have an important role as modulators of the interactions among natural environmental bacterial populations (100). For instance, quinolones alter DNA supercoiling (5), and the expression of many genes, including those involved in virulence, is dependent on DNA supercoiling (44, 112).

More recently, the role of a natural quinolone on quorum sensing in *Pseudomonas aeruginosa* has been demonstrated (258, 299). Quinolone treatment may alter the expression of several bacterial genes. In fact, a decrease in the expression of virulence determinants upon exposure to subinhibitory con-

centrations of quinolones has been described in some cases (62, 361). In contrast, ciprofloxacin and other SOS-inducing antimicrobial agents cause Shiga toxin-encoding bacteriophage induction and enhanced Shiga toxin production from *E. coli* O157:H7 both in vitro and in animal models (187, 424). Therefore, quinolones clearly affect the expression of virulence determinants, but their role as either enhancers or inhibitors of bacterial virulence depends on the model analyzed and must be established for each pathogenic bacterium. On the other hand, resistance to quinolones as a consequence of mutations in bacterial topoisomerases in some cases alters DNA supercoiling (5, 22, 367), leading to changes in the level of expression of several bacterial proteins, including virulence determinants. For instance, treatment of quinolone-resistant *S. aureus* with subinhibitory concentrations of quinolones increases the expression of fibronectin-binding proteins, contributing in part to their emergence and maintenance in clinical settings (47).

Other antibiotics besides quinolones can affect the expression of virulence determinants in different ways. For instance, Suerbaum demonstrated that low antibiotic concentrations increased serum sensitivity in *E. coli* strains protected by the K1 antigen (373), and low macrolide concentrations appeared to reduce the adhesive properties of some gram-negative rods (269). Also, antibiotic treatment may trigger the release of bacterial products, including lipopolysaccharide vesicles (188) and endotoxin (355), that interact with host cells, changing the virulence characteristics of pathogenic bacteria.

Exposure to antibiotics can produce global changes in the metabolism of bacteria, as is possibly the case with *Mycobacterium tuberculosis*. Exposure to low concentrations of antibiotics induces expression of the alternate sigma factor *sigF*, which is involved in the regulation of several genes, including those involved in virulence (263). In light of these data, a role of chemotherapy in the persistence of this important pathogen has been suggested. Recently, it has been shown that tetracyclines are iron scavengers (151). Iron deprivation induces the expression of several virulence-associated proteins in bacteria (224, 243), and it is known that free iron is scarce during infection (409). In this way, tetracyclines might trigger the expression of several bacterial genes just as a consequence of their iron-chelating activity.

The influence of subinhibitory concentrations of antibiotics on virulence is then dependent on the bacterial species-antibiotic combination. The response of resistant strains to antibiotics will be different from that of susceptible ones. For instance, if subinhibitory concentrations of antibiotics reduce the expression of virulence determinants, resistance to drugs (even low-level resistance [29]) would restore the original virulence levels. Major efforts must be made to analyze the effect of antibiotics on the metabolism of antibiotic-resistant and -susceptible bacteria. One such approach is the recently published analysis of isoniazid-induced alterations in *M. tuberculosis* gene expression (7, 415). The authors showed that treatment with this drug produces a dramatic change in the expression of several bacterial genes, some of them related to the drug's mode of action, but others with no apparent relationship to the antibiotic killing mechanism, which may eventually influence the outcome of infection. The current availability of tools for whole-genome expression analysis may allow a better understanding of the role of antibiotics on regulation of bacterial metabolism and thus potentially virulence.

Cross talk between virulence and antibiotic resistance regulons. The environmental regulation of the expression of antibiotic resistance and virulence determinants has already been discussed. The possible cross talk of both types of regulons will now be reviewed. The most relevant example to emerge in the last few years is the effect of quorum-sensing signals on bacterial virulence and the effect of antibiotic resistance on quorum sensing.

Several bacterial species are able to determine the local concentration of bacterial cells, a process known as quorum sensing (37). Quorum-sensing signals trigger the expression of several genes, some of which are involved in bacterial virulence (37, 292, 294). Quite interestingly, it has recently been described that mutations leading to multidrug resistance in *P. aeruginosa* as a consequence of overproduction of the MexAB-OprM efflux pump also affect the quorum-sensing response (117). This response could be due to a direct effect on the extrusion of quorum signals by this MDR pump in *P. aeruginosa* (295). More recently, it has also been described that overexpression of the MexEF-OprN multidrug efflux system affects cell-to-cell signaling in *Pseudomonas aeruginosa* (192).

Since quorum-sensing signals trigger the expression of several virulence-associated genes, such as those involved in production of elastase, rhamolipid, pyocianin, proteases (63), and type III secretion system, antibiotic resistance might strongly influence virulence through the differential processing of quorum-sensing signals in antibiotic-resistant and antibiotic-susceptible bacterial populations. Indeed, recent work in our laboratory has shown that MDR *P. aeruginosa* mutants are impaired in their virulence properties (334).

Conversely, elements with a role in virulence might also be involved in the regulation of antibiotic resistance. As previously stated, iron deprivation induces expression of the MDR efflux pump MexAB-OprM. Also, the expression of MDR determinants can be induced by salicylate (251). Salicylate is a relevant signal molecule in plant-bacteria interactions (351). It is also an intermediate in the synthesis of different siderophores (104, 346) and is itself a siderophore in *Pseudomonas* spp. (346). Bacterial siderophores are relevant virulence factors that are produced during infection (243) as a consequence of the small amount of free available iron that is present in the human body (409). Since *P. aeruginosa* might produce salicylate during infection and this compound induces the expression of MDR determinants, salicylate might thus induce a phenotype of antibiotic resistance for bacteria growing under iron-deprived conditions, such as occur during infection (409).

Mutations in the genes encoding some histidine kinases, which are involved in signal transduction mechanisms in *Streptococcus pneumoniae*, cause a phenotype of tolerance to the killing ability of glycopeptide antibiotics (281), and these mutants are also less virulent when tested in animal models (384). Deletion of the *ciaR* gene, which is involved in regulation of competence and cefotaxime resistance in *S. pneumoniae*, led to a 1,000-fold attenuation of bacterial growth in vivo. Deletion of the *vncSR* gene pair, which is involved in tolerance to glycopeptide antibiotics in this bacterial species (281), also produces growth attenuation, although the effect is smaller. These signal

transduction systems are thus pleiotropic regulators that may influence antibiotic susceptibility, virulence, and even horizontal gene transfer. Therefore, antibiotics may select for variants unable to respond to a number of environmental signals. If that is the case, the environmental signals that induce pathogenicity traits may eventually be altered.

Signal transduction systems also have a role in antibiotic resistance in *P. aeruginosa*. *phoP-phoQ* mutants showing increased resistance to aminoglycosides, cationic peptides, and polymyxin have been described (231, 232). PhoP-PhoQ is a two-component regulatory system that has been studied mainly in *S. enterica* serovar Typhimurium. In this bacterial species, PhoP-PhoQ regulates the expression of at least 40 genes in response to magnesium concentrations (360) and has an important role in both virulence (139, 266) and resistance to polymyxin (155). Although a role of *P. aeruginosa* PhoP-PhoQ in virulence has not yet been demonstrated, it is highly expressed under magnesium starvation (232), which indicates a role similar to that previously described in the case of *S. enterica* serovar Typhimurium. PhoP-PhoQ may then coordinately regulate both virulence and antibiotic resistance in response to the magnesium concentration.

We have addressed some examples of cross talk between antibiotic resistance and virulence regulons. However, genes encoding both types of determinants might also be part of the same regulon. As previously stated, this is the case for the Mar regulon in *E. coli* and *S. enterica* serovar Typhimurium, which is involved in multiple antibiotic resistance (156), superoxide resistance (106, 177), organic solvent tolerance (18), and bile salts resistance (230), among others. The same situation probably occurs for several other MDR determinants, so that the possibility that the physiological signals that bacteria receive during infection might trigger a global response including the regulation of both antibiotic resistance and virulence genes should be carefully analyzed.

THE CROSSROADS OF VIRULENCE, EPIDEMICITY, AND ANTIBIOTIC RESISTANCE

The pathogenic relevance of a bacterium relies mainly on two different properties, virulence and epidemicity (the ability to produce epidemics). A compromise between the two properties is required to cause a major disease because a nonvirulent bacterium obviously will not produce any disease, whereas a nontransmissible bacterium might cause a dangerous disease but only in a limited number of people. Since bacteria are under antibiotic pressure during infection (see above), highly efficient pathogens must be not only virulent and epidemic but antibiotic resistant as well. The problem from the microorganisms' perspective is whether or not the acquisition of one of these traits could have a detrimental effect on the expression of the others. In such a case, an "optimization strategy" will be required. We will review whether the acquisition of these abilities is detrimental to the expression of the others and, conversely, reinforces the possibility of acquisition of a full proinfective repertoire, virulence, epidemicity, and antibiotic resistance.

Are Antibiotic-Resistant Bacteria More or Less Virulent?

When antibiotic resistance occurs as a consequence of a genetic alteration (241) in a housekeeping gene (for instance, those involved in ribosome function, cell wall construction, biosynthetic pathways, and the DNA replication machinery), conventional wisdom suggests that an evolutionarily optimized mechanism has deviated from its functional optimum. Therefore, resistance should have a "direct" cost in bacterial fitness, and resistant organisms should exhibit suboptimal behavior. The location of antibiotic resistance determinants in accessory genetic elements (plasmids, transposons, integrons) probably reduces this "direct" cost, but at the expense of the "indirect" cost of carriage of the elements themselves (8). In both cases, antibiotic resistance should have a fitness cost (13), which might reduce bacterial virulence.

Although it is difficult to find examples of the effect of antibiotic resistance on the virulence of clinical bacterial isolates, epidemiological data support the idea that, in some cases, antibiotic-resistant organisms may show a decrease in pathogenicity. For instance, in urinary tract infections, fluoroquinolone-resistant *E. coli* are easily found in cystitis but are isolated at very low frequencies as causative agents of pyelonephritis (54). Recent studies also suggest that penicillin-resistant *S. pneumoniae* strains may be less pathogenic than susceptible ones, at least in animal models (20, 234), and isolates from cases of bacteremia are frequently less resistant than those isolated from mucosal infections and even in carriers (150, 175). In this case, a possible explanation is that the capsulation involved in serum resistance eventually interferes with acquisition of foreign DNA encoding resistant penicillin-binding proteins.

Nevertheless, the pathogenic process might provide an advantage for the bacteria, such as permitting access to exclusive habitats. For instance, a reduction in the rate of protein synthesis because of a ribosomal resistance mutation may be deleterious for a bacterial organism competing with the wild-type susceptible strain in a rich medium, where the effect of such a deficiency is maximized, while under slow-growth conditions, the difference would be minimal. The expression of genes involved in pathogenicity is frequently regulated by stationary-phase signals (160, 207), so that the slow growth due to a reduction in protein synthesis might eventually trigger the expression of genes involved in pathogenesis. In this case, the growth defect produced by antibiotic resistance could be compensated for by the increased virulence of the pathogen, and bacteria could easily accumulate compensatory mutations which restore their original pathogenic abilities totally or in part (213).

Although antibiotic resistance usually reduces bacterial fitness, the opposite situation might also occur. For instance, the bleomycin resistance gene contained in transposon Tn5 confers improved survival and growth advantage on *Escherichia coli* (55). The increase in the virulence properties of antibiotic-resistant bacteria is a disturbing possibility, because in these cases, revertants that become susceptible by means of mutations and loss of antibiotic resistance plasmids may be at a selective disadvantage relative to the ancestor resistant population, so that the resistant population will prevail even in the absence of antibiotic selective pressure.

In general, antibiotic resistance obviously increases the overall pathogenic potential of the organism in the treated patient, because the susceptible organisms are killed by the antibiotics and the infection does not progress. For instance, the mortality rate of *S. pneumoniae* meningitis in South African children is significantly higher for antibiotic-resistant strains (133, 134). However, in the absence of antibiotics, antibiotic resistance may influence pathogenicity in different ways, depending on the antibiotic resistance determinants involved and the possible accumulation of compensatory mutations.

Are Antibiotic-Resistant Bacteria More or Less Epidemic?

The term epidemic is usually restricted to widespread outbreaks of pathogenic and/or resistant microorganisms. Thus, the actual dimension of the link between transmissibility and resistance may be difficult to evaluate without extensive studies on the population biology of microbes associated with humans. Although the epidemiology of nonvirulent and nonresistant organisms remains to be evaluated, some important observations (163) indicate that in countries such as Sweden, where antibiotic-mediated selection is low, epidemics of antibiotic-susceptible but pathogenic bacteria may be, as in the preantibiotic era, the usual case. Studies of commensal bacteria in wild animals also suggest that the gut flora of wild populations with little human contact (and thus no contact with antibiotics) shows very low levels of antibiotic resistance (288), whereas those from the guts of animals that have been in contact with humans show higher levels of antibiotic resistance (143, 144). On the other hand, the most relevant increases in antibiotic resistance at the local and global levels are due to the spread of a relatively small number of resistant bacterial clones, such as with the "international" resistant clones of *S. pneumoniae* (79, 165, 225), methicillin-resistant *S. aureus* (135, 225, 393), and vancomycin-resistant *Enterococcus faecium* (28, 84). The extreme rapidity with which some of these resistant organisms spread in remote countries suggests that their colonizing potential is nearly optimal for a broad range of hosts.

Several reasons can be given in favor of the existence of an evolutionary link between antibiotic resistance and host-to-host transmission. The diminution in bacterial diversity due to antibiotic treatment will result in overgrowth of resistant bacterial populations that are in the minority under normal competitive circumstances. Antibiotic treatment is then a bottleneck that is crossed by the few antibiotic-resistant bacteria that are present in the population. However, once this bottleneck is crossed, the best colonizers among the remaining antibiotic-resistant bacteria will have an advantage for recolonizing the host. If the challenge is repeated several times, it could be expected that a certain within-host evolution of bacteria to optimize colonization will occur (412). Considering that all members of the host population are very similar in their ability to be colonized by each particular organism, success in colonizing the host will be reflected in a corresponding success in between-host transmission ability. Large numbers of resistant cells within the host should facilitate host-to-host transfer, particularly to other treated hosts. This perspective may have some exceptions. Some bacterial populations are strictly linked to particular environments, including a dependence on other local bacterial populations (41). That situation is not infrequent in mucosa-associated communities. In these cases, the antibiotic-resistant population may remain confined and even disappear under antibiotic therapy just because the other members of the community are eradicated.

The possibility that a resistant population will be enriched under antibiotic treatment thus requires the ability of this species to independently exploit the available habitat. This ability is common among organisms capable of crossing ecological barriers, including the more epidemic ones. In serial-passage experiments with mixed populations, the strain with the greatest number of cells in the transferred inoculum has a selective advantage. The driving force may be within-host competition and selection for increased parasite growth rate (114). In this regard, the ecological abundance of specific clones in natural populations of *Staphylococcus aureus* might be linked to the virulence of such clones (99). The clones should then have been selected because they are good colonizers as well as being highly transmissible and highly virulent (99, 221). This statement is true in general, except perhaps for organisms with high virulence, such as *Mycobacterium tuberculosis*, for which a small inoculum may be sufficient to cause the illness.

Epidemicity ensures constant high multiplication rates that may be needed for the acquisition of resistance. Therefore, these bacteria are simultaneously more prone to becoming resistant, to be selected for by antibiotics, to increase their absolute numbers, and consequently to be transmitted efficiently. There is an obvious corollary: antibiotics should be prescribed (57) in a way that ensures eradication of the bacterial pathogen (as said by Paul Ehrlich, "hit hard and hit early"), as any survival gives the organism an opportunity to evolve and spread in a more efficient way.

The relationship between epidemicity and pathogenicity may also help to modulate the development of resistance. Within-host growth usually induces the expression of virulence determinants in several pathogens, thereby increasing their virulence. Increased between-host bacterial transfer may also increase virulence. It is also well known that serial passage of a given organism among susceptible hosts frequently increases its pathogenic power in the new host, although it is attenuated for the former host (114). Bacterial virulence might then be increased in densely clustered host groups (camps, schools, hospitals), with close contact and high transmission rates, particularly in the early stages of the spread. Antibiotics preferentially exert their selective pressure on clinically ill patients, who are treated more frequently than carriers. If extinction of the pathogen does not occur as a result of treatment, those epidemic strains will tend to be subjected to increased antibiotic pressure, and antibiotic resistance will be more easily achieved.

Although we think that antibiotic resistance usually helps microorganisms in their transmissibility, this is not the case for all. For instance, comparative studies of mutations in animal isolates and experimental in vitro- and in vivo-selected mutants of *Salmonella* spp. suggest that in some cases, a counterselection of highly fluoroquinolone-resistant strains might occur in the field (145). This indicates that fluoroquinolone-resistant *Salmonella* spp. probably have a strong reduction in fitness outside of the host, so that they cannot be maintained in the environment. Alternatively, it could be that the compensatory mutations accumulated during infection (49) are deleterious

for bacteria growing in the environment (see above). The same probably occurs for opportunistic pathogens such as *P. aeruginosa*, *Burkholderia cepacia*, and *Stenotrophomonas maltophilia*. These bacterial species are intrinsically resistant to several antibiotics (307), but, during therapy, mutants with higher levels of resistance and displaying resistance to other antibiotics are selected (140, 378, 392, 422).

It is noteworthy that cross infection is not frequent for these opportunistic pathogens, and the resistant clones selected during therapy are not frequently disseminated. In the case of fluoroquinolone-resistant *Salmonella* strains, this suggests that these resistant opportunistic pathogens are probably impaired in their dissemination in natural environments (mainly water and soil). Of course, as we want to stress throughout this review, this is not a black and white situation, and outbreaks of nosocomial infections due to the transmission of resistant clones have been described. Relevant examples of this situation are vancomycin-resistant *E. faecium* (28), the selection of such antibiotic-resistant bacteria in avoparcin-exposed farms (321), and some outbreaks of infections with multidrug-resistant *Acinetobacter baumanii* (191, 228).

Are Virulent Bacteria More Resistant to Antibiotics?

Because virulent organisms are able to produce clinical symptoms of infection in human and animal hosts and should therefore be exposed more frequently to antimicrobial drugs, the risk of development of resistance is expected to be higher. That is expected to produce a link between higher virulence and higher resistance. Despite this prediction, the opposite is frequently the case. Pathogens such as *Neisseria meningitidis*, *Salmonella enterica* serovar Typhi, *Shigella dysenteriae*, *Bordetella pertussis*, *Leptospira icterohaemorragica*, and *Brucella melitensis* are rarely resistant to antibiotics. Some reasons can be given for this phenomenon.

First, as stated previously, the possible cost (in fitness) of antibiotic resistance should be tolerable by the bacterial host. Usually this tolerance involves the acquisition of cost-compensating mutations that must be tolerated as well. This is not a critical problem for many opportunistic pathogens that are able to survive in various alternative environments. But in several highly specialized pathogens, the genetic structure cannot be extensively modified by mutation without risk of local extinction as a consequence of an increased number of lethal mutations (14). Second, in contrast to less-specialized pathogens, some of these highly virulent organisms have probably evolved in protected environments less open to competition and therefore less exposed to natural antibiotics. Third, the absolute number of many pathogens is reduced compared to their commensal counterparts, so that its chances of being exposed to antibiotics and acquiring antibiotic resistance should be lower. This view has been proposed as an explanation for the differences in the ability to develop mutational resistance to penicillins in *S. pneumoniae* and the viridans group streptococci versus *S. pyogenes* (28). Fourth, the particular niche occupied by highly pathogenic organisms may be excluded or almost excluded from the action of antimicrobial agents (see below), so that they are not subjected to antibiotic selective pressure.
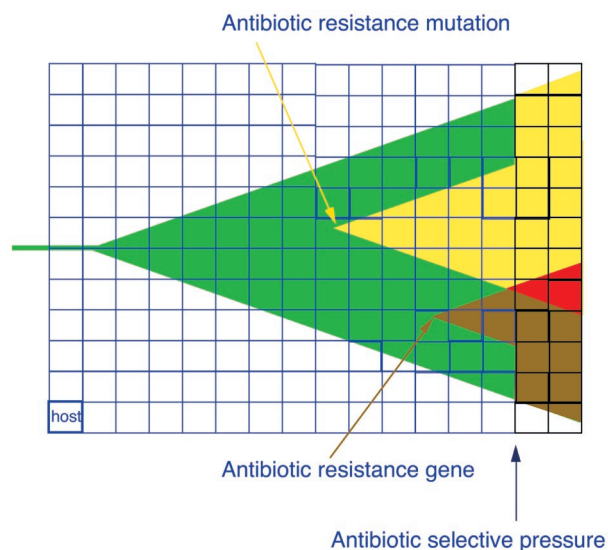

FIG. 3. Epidemic bacteria can easily acquire an antibiotic-resistant phenotype. An epidemic bacterial strain (green) spreading between hosts (each individual blue square) increases its population size, facilitating the emergence of antibiotic-resistant mutations (yellow arrow) and the acquisition of resistance genes by horizontal transfer (brown arrow). Acquisition of resistance is immediately amplified by the epidemicity of the strain, even in the absence of antibiotic selection (resistant variants, yellow and brown). Interactions between different mechanisms of resistance may occur, eventually leading to multiresistance (red). If antibiotics are almost ubiquitously present in the hosts, as in an intensive care unit (black squares), the spread of the resistant bacteria is favored.

Are Epidemic Bacteria More Resistant to Antibiotics?

Epidemic bacteria are considered here as those able to spread in an efficient way between hosts in a short period of time (note that we do not mention whether epidemic bacteria are pathogens or not). This property is also frequently expressed as organisms having a "high reproductive rate" (R_0) (19, 283). By definition, epidemic bacteria are frequently encountered in a large number of individuals and thus reach high population sizes throughout the host community. Because these populations frequently face variable environments in terms of antibiotic selective pressure (patients under therapy and not, different therapeutic regimens), the chances for acquisition of antibiotic resistance increase (32). Additionally, bacteria with a high dispersal (migration) rate are in contact with more types of microorganisms occupying different ecological habitats from which antibiotic resistance genes can be acquired by horizontal transfer. These characteristics make epidemic bacteria more prone to acquire antibiotic resistance. Once an antibiotic resistance phenotype is acquired, an epidemic microorganism can be widely disseminated. In addition, antibiotic selective pressure in treated patients will convert such an organism into a predominant clone in this bacterial species (Fig. 3). This is probably the cause of the worldwide distribution of antibiotic-resistant clones of species such as *S. pneumoniae* (see above).

Epidemicity implies that the microorganism can colonize a large number of hosts, so that the overall number of bacterial cells to which selection can be applied is also large. Thus, the

probability of selecting an antibiotic-resistant mutant is higher than in the case of nonepidemic microorganisms. On the other hand, if an epidemic organism becomes resistant in one host and is transferred to other hosts, the use of antibiotics in each treated host ensures the removal of the susceptible bacterial populations and its replacement by the epidemic population. At this stage, the antibiotic-resistant microorganisms increase in number, and all the possible variants within this population compete for establishment in the novel hosts. As a result of this competition, the best colonizers of the epidemic, antibiotic-resistant microorganism are now selected. Selection of epidemic and antibiotic-resistant bacteria is thus based on a feedback mechanism which links the two properties, unless the cost of antibiotic resistance severely impairs the effectiveness of host-to-host spread (see above). This cost will depend on the specific bacterial species-antibiotic resistance mechanisms. In this way, studies which integrate virulence properties and the behavior of antibiotic-resistant microorganisms in the environment are needed to establish the best protocols for avoiding the spread of virulent antibiotic-resistant bacteria.

RESISTANCE AND VIRULENCE: AN INTEGRATED VIEW

The human host has acquired several different mechanisms in the course of evolution for fighting infections, including the immune system, fever, production of antimicrobial peptides (136, 174, 397), and iron-scavenging (376), among others (210). Another mechanism of defense against colonization and further infection is the presence of an indigenous microflora which has coevolved with its host (41). This defense constitutes the very first biological barrier against subsequent colonization and infection with external potentially pathogenic microorganisms. Very recently in terms of evolutionary time, a new line of defense has been added to the armamentarium of humans in their fight against infection, that being antibiotic therapy.

Based on the effect that they produce on infectious bacteria, pharmaceutically produced antibiotics play the same anti-infectious role as antimicrobial compounds produced by either the host or its allied microbiota. All these types of substances are able to inhibit bacterial growth and thus constitute a part of the defense of the human host. The only difference between these substances resides in the fact that conventional (industrial) antibiotics are a product of the so-called "cultural evolution" (123), whereas the other mechanisms for defense against infections are the result of the natural "genetic evolution" of the human species. In opportunistic infections, where the genetic defense strategy is severely impaired, the only line of defense that remains is the antibiotic cultural defense. With this approach, antibiotic resistance is hard to distinguish from other mechanisms involved in microbial pathogenicity.

Resistance as a Colonization Factor in the Treated Host

Acquisition of an antibiotic resistance phenotype will have two effects on bacteria during colonization and infection of the treated host. First, resistance is required for evading cultural host defenses (in this case, antibiotic treatment). Therefore, it is equivalent to other systems for evading other host defenses, such as production of siderophores (88), resistance to oxidative stress (68, 106, 284), resistance to defensins (136, 174), and resistance to bile salts (382, 396) for intestinal pathogens. These characteristics are needed, although not sufficient, for the development of infection. Second, resistance in the presence of antibiotics in the treated host will favor colonization. As stated previously, the very first line of defense against infections is the indigenous microflora (41), which form a colonization barrier against exogenous microorganisms. However, this microflora is severely affected by antibiotic therapy. Therefore, antibiotic therapy debilitates the defenses of the host by altering the protective biological defenses. Only if the infecting microorganism is resistant to antibiotics can it make use of this advantage for better colonization of the host.

We might thus conclude that antibiotic treatment promotes further colonization and infection by antibiotic-resistant bacteria which otherwise may not be able to effectively compete with indigenous microflora. In this way, antibiotic resistance is a relevant colonization factor for the microorganisms interacting with the treated patient. In fact, epidemiological studies have demonstrated that antibiotic therapy is a relevant risk factor for colonization by antibiotic-resistant bacteria such as *S. pneumoniae*, methicillin-resistant *Staphylococcus aureus*, *Stenotrophomonas maltophilia* (108), *Acinetobacter baumannii* (307), and *Campylobacter jejuni* (356).

Opportunistic Infections

Opportunistic infections occur in individuals whose natural defenses against bacterial infection are severely impaired (42, 182, 307). In the preantibiotic era, these people were mainly infected by microorganisms belonging to their own microflora. Currently, even when the genetic defense has collapsed, the second line of defense, antibiotics, keeps these hosts free from infection, but obviously only if antibiotic-susceptible organisms are involved. As research on antibiotics has been focused for years on frequent human pathogens, many antibiotics are still active on microbes associated with humans. Because of this, many opportunistic pathogens today come from the environment, with one of their more salient characteristics being high intrinsic resistance to a broad range of antibiotics (307). Indeed, it is this ability that allows opportunistic pathogens to invade the compromised host under antibiotic therapy, since environmental opportunistic pathogens do not need to harbor specific pathogenicity determinants for humans (as the natural defense has been broken), but only antibiotic resistance.

In fact, opportunistic pathogens are unable to produce infections in nondebilitated patients. Therefore, environmental and clinical isolates of opportunistic pathogens such as *P. aeruginosa* (127, 323) and *Stenotrophomonas maltophilia* (40) are genetically indistinguishable. Analysis of two collections of *P. aeruginosa* isolates from environmental and clinical settings has demonstrated that they were functionally equivalent in traits relevant for both their pathogenic (host-oriented) and biodegradative (environment-oriented) abilities (10).

The healthy host is quite an "extreme" environment in terms of stringent growing conditions for microorganisms. Although few bacteria have evolved to efficiently colonize this environment, many more are potentially able to grow using the resources of human body (for instance, those involved in corpse decomposition and putrefaction). In other words, the dead
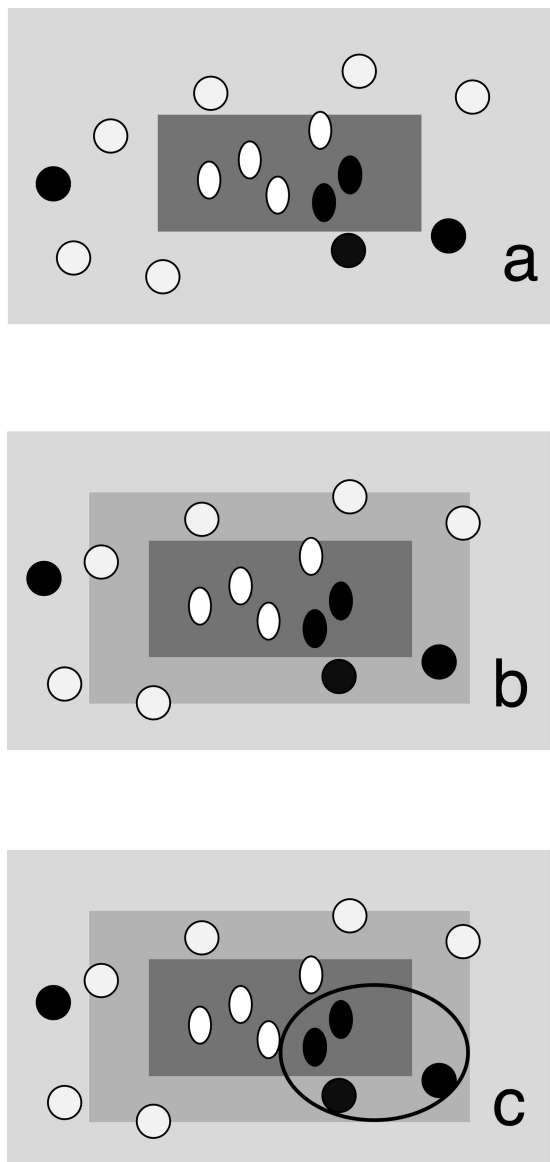
FIG. 4. Colonization space of opportunistic pathogens. Colonization space can be defined as the combination of the different physicochemical parameters (including the space) in which an organism can survive. In this regard, stringent growth conditions (for instance, absence of oxygen) reduce the ecological space. As discussed, conditions for infection are quite stringent, so that only a few bacteria can colonize the host's ecological space (dark gray square) compared to bacteria that colonize the environment and, eventually, the dead host (lightly shaded square). In the case of the sick host, however, stringency for colonization is lower, so that some environmental bacteria can now colonize this larger ecological space (intermediately shaded square in panels b and c). These bacteria with an environmental origin are opportunistic pathogens. Once infected with these bacteria, the host is usually treated with antibiotics. Again, antibiotic treatment restricts the ecological space so that only antibiotic-resistant bacteria can grow under these conditions (oval in C). In all cases, small ovals indicate human-associated bacteria and small circles indicate environmental bacteria. White, antibiotic-susceptible bacteria; black, antibiotic-resistant bacteria.

body implies a rupture of some ecological constraints of the living one. Also, because conditions for bacterial growth are less stringent in the ill host (Fig. 4), a greater variety of bacteria can eventually produce an infection. However, when antibiotic treatment is begun, there is a new ecological constraint on the growth of the bacteria, the antibiotic, so that under these conditions only antibiotic-resistant bacteria will be able to maintain the infection. Finally, because opportunistic pathogens are not the result of evolution of an infectious bacterial species in contact with the host, the term "emergent pathogens" has been inappropriately used for their description. These organisms, having acquired the ability to colonize different habitats, can infect the sick host. Their selection is the result of cultural human evolution, since antibiotic treatment has selected a previously naturally resistant bacterial species. The replacement of traditional opportunistic pathogens with novel, more resistant ones has occurred over the last few years (307). Thus, antibiotic resistance linked to antibiotic therapy might be considered the primary factor influencing the selection of such novel antibiotic-resistant opportunistic pathogens.

Does Virulence Affect Exposure to Antibiotics?

As previously stated, virulent organisms producing clinical symptoms are more frequently treated with antibiotics than nonvirulent ones, and therefore virulence should increase antibiotic exposure. Antibiotic treatment will affect both the infecting bacteria and indigenous microflora. However, pathogenic bacteria will be treated as soon as they are detected (and their presence suspected from clinical symptoms), and thus antibiotic selective pressure is exerted quite frequently on these organisms. Indigenous microflora are challenged with antibiotics only as an unwanted side effect of the therapy directed against the pathogens, so that most time is maintained without antibiotics. Nevertheless, several bacterial pathogens may behave as commensals over long periods of time.

Many bacterial infections are controlled by the host in many cases without clinical symptoms (210). In this case, even pathogenic organisms may not be frequently challenged by antibiotics. In some cases, host lesions induced by pathogenic microbes may occur but with few if any clinical symptoms (subacute infections). In these cases, these organisms with "low virulence" will also be less exposed to antibiotics. One might suggest that antibiotic therapy could be considered a cost and a penalty associated with virulence. If this were the case, it is conceivable that some pathogens could evolve to low pathogenicity after prolonged use of antibiotics in human populations. In general, close relatives to pathogenic bacteria are now members of the normal flora. Are they the result of the evolution from pathogen to harmless variant to escape host defense mechanisms?

In particularly severe infections, the host may quickly die, reducing bacterial exposure to antibiotics. Once the host dies, the microorganisms may either die as well (if they need to grow inside the host) and be released into the environment, where the antibiotic pressure is low. It is thus possible that the most virulent bacteria are perhaps less exposed to antibiotics than nonvirulent ones. We can conclude that bacteria with intermediate levels of bacterial virulence have a greater probability of being exposed and developing resistance to antibiotics than
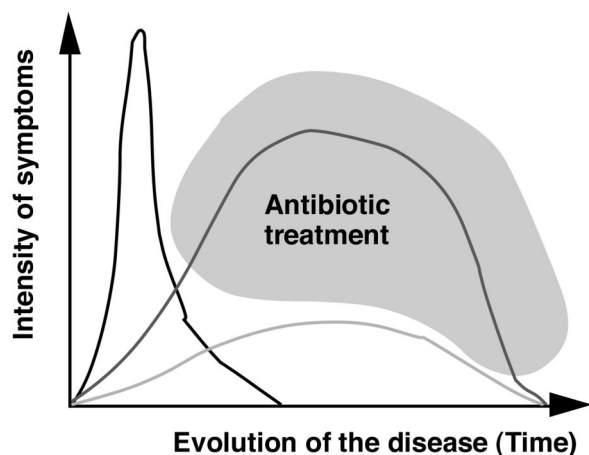
FIG. 5. Infectious bacteria under risk of antibiotic treatment. As stated in the text, in some cases infections are extremely acute (black line), so that the host is damaged (and even killed) before an antibiotic treatment is implemented. Other infections present no symptoms and have subclinical manifestations, so that they are not treated even if they occur for long periods of time (light gray line). Only infectious bacteria that produce clinically relevant symptoms and are present long enough to be treated (dark gray line) are under antibiotic selective pressure.

less virulent and highly virulent organisms (Fig. 5), in the first instance because they are not always detected and thus not treated, and in the second, because the host dies without giving them enough time for the development of antibiotic-resistant variants.

Finally, epidemic organisms may evade a short antibiotic exposure because, by definition, they move to another host (usually nontreated) more easily than a nonepidemic microorganism. Thus, mechanisms facilitating between-host transfer may in some cases reduce exposure to antibiotics, particularly if the organism is not highly virulent. In the long run, however, epidemic organisms will confront higher levels of antibiotics, as the hosts are treated after the infection is detected (see above).

In host spatial location and exposure to antibiotics. The location of microorganisms in tissues during infection is an important factor in their exposure to antibiotics. In some cases, organisms are refractory to treatment even if they are classified as susceptible by standard in vitro tests (242). Virulent organisms may invade spaces in which the achievable concentration of antibiotic is low, such as in otitis, sinusitis, and meningitis (186). Also, the pharmacodynamic properties of antibiotics influence in a critical way the inhibitory and killing effects of these drugs. For instance, if a given concentration of a beta-lactam is not maintained in contact with the organism over a critical period of time, the antibacterial effect (predicted by the AUC/MIC ratio and time above the MIC) will be reduced.

In other cases, there is a critical concentration of antibiotic (C_{max}) that is affected by changes in the medium during infection. For example, low pH is characteristic of inflammatory fluids as well as some intracellular compartments (353). Several antibiotics are less active in acidic conditions, so that the environment of the infection site may contribute to lowering the antibacterial effect of antibiotics (193, 226). As previously discussed, several bacterial pathogens can invade host cells and

are thus less exposed to antibiotics (395). Biofilms (see above) also produce an antibiotic resistance phenotype due at least in part to the structure of the biofilm, which precludes the efficient transfer of antibiotic to all the bacteria present in the biofilm (83, 370). More recently, it has been described that resting cells are as resistant as bacteria growing in biofilms, and the effect of biofilms on antibiotic susceptibility might be due to a stationary-like growth phase of the bacteria growing in the biofilms (364)

Obligate intracellular pathogens such as *Chlamydia*, *Lawsonia*, and *Rickettsia* spp. are infrequently resistant to antibiotics. For these pathogens, the MICs of antibiotics such as tetracycline and erythromycin that are distributed either extracellularly or in intracellular compartments that are different from those in which bacteria are located have remained constant over decades (259) This suggests that some intracellular compartments serve as refuges against antibiotics. It is obvious that intracellular location also prevents easy acquisition of resistance elements such as plasmids and transposons, from other organisms. These elements may occasionally be present in bacteria, as *Coxiella* and *Listeria* spp., which do not have an absolute requirement for an intracellular location for growing. Other obligate intracellular pathogenic organisms such as *Brucella* spp. are infrequently resistant to antibiotics and rarely contain plasmids.

Comparison of the genomes of different bacterial species has demonstrated that in organisms with small genomes, horizontal gene transfer is very infrequent (15, 285). It has been suggested that because of the extreme specialization of the small genome of intracellular organisms, any new gene or gene modification may disrupt the delicate equilibrium of vital genes, leading to extinction of the microorganism (113, 286). In other words, resistance genes might reduce the viability of intracellular organisms. Moreover, the altered metabolism of these organisms might reduce their ability to utilize resistance genes. For instance, the energy needed for expression of resistance to tetracyclines and macrolides by efflux mechanisms may not be accessible in organisms dependent on host cell energy sources (259). In general, intracellular bacteria might tolerate only mutational changes that lead to energy-independent mechanisms of resistance and not reduce intracellular fitness.

The possibility that some bacteria could have developed mechanisms of intracellular invasion in the environment, as an escape from natural antibiotics, cannot be discarded. Facultative intracellular pathogens such as *Legionella* spp. have environmental protozoa as natural hosts, and the pathogenicity of these organisms in humans may only reflect an accidental change leading to the invasion of another type of eukaryotic cell (161). Something similar may have occurred in *Listeria*. Interestingly, acquired antibiotic resistance is a very unusual event in both genera, which indicates that intracellular location is a effective way to escape from antibiotics.

In summary, we want to stress that the location of an organism in a particular body site is influenced by its colonizing and pathogenic abilities. Therefore, these properties determine the cell-antibiotic interaction. On the other hand, this interaction is critical for the eventual development of antibiotic resistance.

Normal microbiota versus pathogenic bacteria. The normal microbiota is the first line of defense against infections due to its role as a colonization barrier (41). However, once coloni-

zation occurs, normal bacterial flora might also protect virulent bacteria from antibiotic action. Protection of a susceptible population by a resistant one has been described in vitro for beta-lactam antibiotics (30). Beta-lactamase-producing bacteria rapidly inactivate beta-lactam antibiotics. If a mixed population of beta-lactamase producers and nonproducers is exposed to beta-lactams, the nonproducers will begin to die. However, once the antibiotic is inactivated by the beta-lactamase producers, the remaining susceptible bacteria that have not been killed by the beta-lactam can grow. This effect is frequently seen in the laboratory, when ampicillin-susceptible colonies exhibit satellite growth around an ampicillin-resistant clone in an ampicillin-containing plate.

It has been demonstrated that minute amounts of beta-lactamase-producing strains can protect fully susceptible strains from the action of beta-lactams (30). During infection, the same situation might occur in vivo. Antibiotic-resistant populations, either as normal colonizers or in the case of coinfection by different bacteria, can then protect virulent, susceptible bacteria from antibiotic action. This effect has been claimed as relevant when beta-lactam therapy failed to eradicate fully susceptible bacteria from colonized environments (66).

Normal microbiota can also contribute to the acquisition of antibiotic resistance determinants by bacterial pathogens because in vivo transfer of antibiotic resistance determinants has been reported (248). Thus, acquisition of antibiotic resistance genes by normal, nonpathogenic microbiota is a concern because the final outcome of antibiotic resistance in bacterial pathogens might be compromised. Indeed, the possibility of maintaining hospitals with a reduced proportion of resistant bacteria depends on the local reduction the antibiotic selection, but also on the dilution of the resistant organisms of the hospital caused by the admission of patients from the community with susceptible normal flora (222).

POPULATION STRUCTURE

The population structure of clinically relevant bacteria is an important epidemiological topic (212). If only one or a few clones of a bacterial species are relevant for infection and antibiotic resistance, efforts must concentrate on these clones. Also, preventive strategies can be more easily designed if the prevalence and spread of the various clones involved in disease are known. A more comprehensive analysis of the infection must involve the study of bacterial populations from both the ecological and evolutionary perspectives. This analysis requires the use of molecular biology tools for analyzing the structure and evolution of bacterial pathogens.

Bacterial Variation Facing Stressful Environments

As was stated above, bacterial adaptation is essentially the consequence of mutation and gene recombination. Mutational events are particularly important when a large population of cells is confronted with critical, abrupt, and nonspecific changes in the environment, eventually permitting rapid but not always optimal adaptation. In fact, selection of bacteria with a hypermutator phenotype (see below) has been suggested to be a good adaptive strategy to sudden environmental

changes (381), and mutational adaptation requires large bacterial populations (381), as this evolutionary strategy implies the death of most members of the population (246). On the other hand, recombination allows the acquisition of several different traits in a single step (153).

In this regard, horizontal transfer of genes and even large chromosomal regions is frequent in all bacterial species (101, 176), contributing to the acquisition of several physiological functions in a single step (see above). However, recombination between different strains of the same bacterial species occurs frequently in only a subset of bacterial species (1, 79, 122) that are highly specialized to grow in constant and frequently in unique habitats (such as *Helicobacter pylori, Neisseria meningitidis, Neisseria gonorrhoeae, Haemophilus influenzae*, and *S. pneumoniae*). In these cases, recombination between members of the same species is important for combining the beneficial mutations present in the entire bacterial population and eliminate the deleterious ones. In fact, it has been stated that a high recombination rate could be selected when several mutations (present in different members of the population) are needed for adaptation (118).

Bacterial adaptation can increase under conditions of stress such as infection and antibiotic selective pressure. In the last few years, it has been shown that bacteria have stress-regulated systems which allow them to mutate at a higher rate under stress conditions. Two different situations are relevant for increased mutation rates in vivo in bacterial populations.

Stress-induced transient increases in mutation rates. Bacteria increase their mutation rate as a consequence of stress, such as starvation (185, 348), antibiotic challenge (9, 318), and possibly during infections; this process is termed adaptive mutation (129, 348). A situation of stress triggers a bacterial response that involves changes in the expression of several proteins. These changes are a consequence of the recognition by the bacteria of "stress signals" that activate a global response based in the concomitant transcriptional regulation of several genes (343, 383, 421). Some aspects of these responses are specific to the type of stress involved, but other changes are common to different stress situations.

Little is known about the bacterial responses to pathogenic and antibiotic-induced bacterial stresses. However, it is conceivable that these types of stress trigger responses similar to those in other bacterial stress situations. For instance, treatment with antibiotics at bacteriostatic concentrations provokes a stationary phase-like response, and it has been demonstrated that subinhibitory concentrations of antibiotics targeting the ribosome induce the synthesis of either heat shock or cold shock proteins, depending on whether the A site of the ribosome was empty or occupied (45). In any case, adaptive mutation to stress is a regulated phenomenon by means of which bacteria can increase their mutation rate severalfold (327). The primary factors in this process are stress-responsive, error-prone DNA polymerases V (*umuCD*) and IV (*dinB*), whose expression transiently increase the rate of bacterial mutation (310). Eventually, bacterial stress over the pathogenic process may increase the probability of emergence of antibiotic-resistant mutants; conversely, antibiotic stress may increase bacterial adaptation to the host.

Hypermutable (mutator) strains. Hypermutation can be the consequence of a regulated increase in mutation rates (see

below) or the presence of subpopulations of "mutator" bacteria within "normal" bacterial populations (252, 386). Some works have demonstrated the presence of such mutator populations in infecting bacteria (67, 208, 287). These cells have mutation rates that can be increased as much as 10,000-fold, usually as the consequence of inherited defects on the methyl-directed mismatch repair system (380). These strains should have increased abilities to survive antibiotic challenge and antibacterial host defenses. Among strains lacking the methyl-directed mismatch repair system, the ability to recombine (homologous recombination) should increase (253), which may facilitate the acquisition of both antibiotic resistance and bacterial virulence in the same organism.

Mutator strains are supposed to be less fit than wild-type strains outside the stress situation (adaptive bottleneck). Induction of a transient hypermutation under stress serves to generate mutants which can escape from transient stress situations, whereas stable mutator strains are fixed in the populations only when bacteria are subjected to frequent bottlenecks. This situation probably occurs in chronic infections, such as those associated with cystic fibrosis. In fact, *P. aeruginosa* isolates from cystic fibrosis patients frequently present a mutator phenotype, a characteristic feature that does not occur in *P. aeruginosa* isolates obtained from blood and sputum cultures of non-cystic fibrosis patients (287).

Recently, the fitness of mutator *E. coli* strains has been analyzed in an in vivo model system (146). A high mutation rate was shown to be beneficial because it allowed faster adaptation. However, the benefit disappeared once adaptation was achieved, because mutator strains accumulated mutations that, although neutral in the analyzed model system, can be deleterious in a secondary environment. Thus, mutator strains may have short- and long-term advantages that justify high competitiveness of mutator bacteria during infection, but low competitiveness during transmission and recolonization of other hosts.

In summary, stressful growth conditions in the host, particularly under antibiotic therapy, are important elements for the evolution of bacterial populations, influencing gene transfer and increasing mutation rates. Stress associated with the pathogenic process and antibiotic exposure not only increases bacterial variation, but also strongly selects particular bacterial variants. In this sense, pathogenesis and antibiotic exposure can be considered "accelerators" (31) of bacterial evolution.

Clonal Structure

Molecular epidemiology has demonstrated that a few clones may account for the worldwide dissemination of virulent and antibiotic-resistant bacteria for several bacterial species. This view of the genetic structure of bacterial species is known as the clone concept (345, 368). Further analysis has demonstrated that the degree of clonality varies depending on the species analyzed. In that way, pathogenic species such as *E. coli* and *Salmonella* spp. are highly clonal, whereas species such as *Neisseria gonorrhoeae* present a very high rate of recombination and have a panmictic (species that are not subdivided into discrete phylogenetic lineages [385]) genetic structure (357). Even for microorganisms with a predominantly clonal structure, analysis of complete genome sequences has demon-

strated that horizontal transfer due to recombination is frequent in bacterial populations (204).

The high rate of recombination in several bacterial species might put in question the validity of the epidemiological analysis of clinically relevant bacteria under the clonal structure approach. However, even for bacteria for which recombination occurs very frequently, this approach can be extremely useful. When analyzing the genetic structure of these bacterial populations, evolution can be traced over time, following the routes of transmission. For highly epidemic species such as *Neisseria meningitidis*, clones emerge that disseminate further worldwide. Vaccination against the most frequent serotypes may produce the replacement with less common ones (see below). Also, the replacement of antibiotic-susceptible with antibiotic-resistant clones has occurred, and these replacements are easily followed by using molecular typing methods (225). In this case, a transient "quasi-clonal" population can be observed at a fixed time depending on the geographic localization (148).

This situation is the consequence of the emergence of clonal lineages which are more proficient in their epidemicity, virulence, and antibiotic resistance than others. The more successful lineages will then predominate. However, once they become the predominant population, they will produce novel divergent lineages as a consequence of recombination with other clones and may then expand in different environments. The presence worldwide of different environmental habitats for bacterial populations guarantees this divergence, as has been demonstrated in different model systems (313).

The clonal hypothesis is also relevant for understanding the evolution of genetic elements that are transmitted in bacterial populations. In fact, the variability of antibiotic resistance genes with clinical relevance is not very high, which indicates a "clonal" origin for several. Thus, most antibiotic resistance genes currently acquired by horizontal transfer were originated by transfer of an ancestor gene to a single or a few lineages of bacteria and then disseminated among bacterial populations. For instance, it has been demonstrated that the gene *mecA*, responsible for resistance to methicillin in *S. aureus*, was acquired only once by this bacterial species (196). Something similar has probably occurred for widely distributed genes such as plasmid-borne beta-lactamase genes of the TEM group (71).

Even for highly recombinogenic bacterial species, a clonal origin of their genetic resistance determinants can eventually be traced. An example is the evolution of the genes encoding the synthesis of penicillin-binding proteins in *Neisseria* and *Streptococcus* spp. The analysis of *pbp* genes from different isolates has demonstrated that they are very uniform among penicillin-susceptible strains of *N. meningitidis, N. gonorrhoeae*, and *S. pneumoniae*, whereas those from penicillin-resistant strains consist of a mosaic structure that arose from the horizontal transfer of blocks of DNA from other, more naturally penicillin-resistant, commensal species by genetic transformation (79, 365). Analysis of beta-lactam-resistant populations has demonstrated that these novel *pbp* genes have occasionally been selected, as single gene "clones," and afterwards, either the antibiotic-resistant bacterial clone or the *pbp* "resistant gene clone" disseminates and evolves further among the bacterial population.

The same notions applied for analyzing the evolutionary biology of bacterial populations can be applied when analyzing

accessory genetic elements (mainly plasmids and transposons). The most successful plasmid will be one with a good compromise between plasmid "epidemicity," defined as the ability to transfer between different species (number of different species or strains to which the plasmid can be transferred), "infectivity," defined as the ability to infect a specific host (frequency of entrance in the bacteria); and presence of selective traits (see above). As discussed above, antibiotic resistance may reduce bacterial fitness (13), and the same occurs with the acquisition of plasmids (8). If the fitness reduction is too high, the resistant bacteria will be unable to compete with the wild-type susceptible ones. Thus, most probably, we only detect those antibiotic resistance genes that do not produce an unaffordable fitness reduction on bacteria.

We think that there are probably many more antibiotic resistance genes than those currently detected in bacterial populations. As stated previously, accessory genetic elements of clinically relevant bacterial populations also follow the rules of the clonal hypothesis. The same *vir* plasmids are found in all members of a single *Yersinia* species and are quite similar when one species is compared to another (306). This indicates both the clonal origin for these determinants and their successful maintenance in *Yersinia* populations for very long periods of time. Also, dissemination of epidemic antibiotic resistance plasmids is very frequent, and the acquisition of novel genetic determinants by a clonally originated plasmid has been documented (31, 98). In fact, analysis of bacterial collections from the preantibiotic and postantibiotic eras demonstrated that they contain plasmids belonging to the same families, although the ones from the preantibiotic era do not contain antibiotic resistance genes (95, 171). These results indicate the ability of plasmids to evolve as evolutionary units through the acquisition of antibiotic resistance determinants.

The clonal hypothesis is then a useful tool for analyzing relationships between bacterial strains as well as mobile elements and even between genes involved in clinically relevant phenotypes. The use of this analysis of the lineages of such different elements can give a complete picture of the evolution of bacterial pathogens in response to novel selective pressure (such as antibiotic treatment). This approach is also relevant from the epidemiological point of view and can offer new light for understanding the future evolution of bacterial pathogens in terms of virulence, epidemicity, and antibiotic resistance.

The application of the clonal hypothesis to the study of both organisms and genes will eventually serve to explore the hypothesis of possible convergent evolution of antibiotic resistance and bacterial pathogenicity, eventually leading to increasing numbers of organisms that are both resistant and virulent (see below).

Resistance and virulence decrease bacterial diversity. Virulent bacteria must face several environmental changes over the course of infection. To infect the host, bacteria must enter the organism, traverse through different epithelia, avoid the action of different host defense mechanisms against infection, and finally reach the target organ(s), where they produce the disease (Fig. 1). It is intuitively appealing that the challenge of bacterial populations by these successive selective bottlenecks should reduce genetic diversity, at least in the short term.

Changing environments are very stringent conditions for evolutionary adaptation, so that only a few organisms can survive those changes. As stated in the legend to Fig. 1, this is the case for the process of infection. The diversity of commensal bacteria in humans is extremely high, which shows that many different bacteria are adapted to grow on each of the different host surfaces (intestine, skin, oropharynx). On the contrary, the diversity of virulent bacteria is much lower (357). Virulent bacteria need to traverse and eventually colonize different environments as well as survive stringent and changing conditions (see Fig. 1). The requirement for multiplication under all these stringent conditions might explain the low diversity of very specialized pathogens. A release from some of the stringent conditions required for growth in the healthy host will increase the possible diversity of potential pathogens. In this regard, a diverse array of opportunistic pathogens are able to infect the immunosuppressed and severely ill host, which presents a much less stringent environment (see above).

A stringent selection also occurs for antibiotic resistance. In this case, rapid changes in the environment, produced by antibiotic exposure, rapidly lead to lethal conditions of selection. Only resistant clones will survive, replicate, and subsequently colonize the previously complex ecosystem. In fact, it is well known that many antibiotic treatments reduce the bacterial diversity of the human-associated microflora. Selection is even stronger with conditions of combined and successive antibiotic therapy, because bacteria are challenged by simultaneous and consecutive lethal bottlenecks. Therefore, only a few types of bacteria will survive, and diversity will be strongly reduced as a consequence of the treatment.

Taxonomic Implications of Changes in Resistance and Virulence

The development of antibiotic resistance and the emergence of new pathogens have two implications for the taxonomy of microorganisms.

Misclassification as new species. The phenotypic changes associated with the acquisition of an antibiotic-resistant phenotype may alter the characteristics of the well-known human pathogens, producing mistakes in the classification of these microorganisms. For instance, optochin-resistant *S. pneumoniae* (300) could be easily classified with the viridans streptococci (58), and penicillin-resistant pneumococci manifest major changes in peptidoglycan structure (138). If classic approaches to phenotypic identification are used without knowledge that these bacteria are antibiotic-resistant derivatives of pneumococci, a new species defined as "an antibiotic-resistant gram-positive organism, very close to *S. pneumoniae* but with some different features, such as resistance to beta-lactams and a characteristic peptidoglycan structure" could have been described.

Emergence of novel microbial species. The emergence of novel virulent species may occur by the acquisition of pathogenicity islands, which changes the pathogenic characteristics of the bacterial species. For instance, we know now that *Y. pestis*, the cause of plague, is a recently emerged clone of the much less virulent *Y. pseudotuberculosis* (2). Also, acquisition of a new host specificity could lead to novel species, such as occurs with the human immunodeficiency virus (for instance, from chimpanzees to humans [137]) and several mycobacteria (from ruminants to humans [17, 53]). In fact, it has been

TABLE 3. Factors that might influence the future evolution of virulence and antibiotic resistance

Factor	Outcome
Sanitation and hygienic measures	Reduction in the number of infections
Preservation (eventually bioremediation) of ecosystems	Maintenance of microbial diversity
Human demographic changes	Increased number of host-pathogen contacts
Environmental changes	Changes in the geographic localization of microorganisms
Commercial interchanges	Wide spread of microorganisms
Increased number of elderly people	Increased number of opportunistic infections
Aggressive medical technologies	Increased number of opportunistic infections
Intensive farming	Reduction in microbial diversity; selection of antibiotic-resistant bacteria by antibiotics and food additives
Diseases that alter host response to infection	Increased number of opportunistic infections
Novel antimicrobial strategies	Better treatment of infections
New vaccination technologies	Prevention of infections
Bioterrorism/biological war	Spread of old and/or novel pathogens

suggested that speciation of *M. tuberculosis* occurred 15,000 to 20,000 years ago (183) at the time of ruminant domestication.

Finally, new bacterial species (and strains) for the moment unknown might emerge linked to novel diseases. For instance, enterohemorrhagic *E. coli* (131) has been recognized as a relevant health problem just in the last two decades. However, the large divergences found upon comparison of the sequence of the whole genome of the enterohemorrhagic *E. coli* strain O157:H7 (297) with the nonpathogenic laboratory strain K-12 (52) demonstrates that these strains diverged a long time ago.

FUTURE EVOLUTION IN AN ECOLOGICAL PERSPECTIVE

Major Factors

The future evolution of bacterial virulence is expected to be shaped (Table 3) by at least a dozen major factors.

Sanitation and hygienic measures (mainly drinking water and sewage management). The rise in the world population coupled with demographic changes (megacities, displacement of the population from the countryside to the cities, increased numbers of refugees) produces an increased requirement for noncontaminated water reservoirs. Control strategies must be implemented to guarantee the quality of water and food resources used in agriculture and animal and human consumption in order to prevent the emergence and dissemination of potentially relevant novel diseases.

Preservation and eventual bioremediation of normal ecosystems in human, animal, and environmental microbiota. Industrial contamination produces severe changes in natural ecosystems. Heavy metals, pesticides, solvents, and even antibiotics are among the most conspicuous contaminants. The effect of this type of contamination on indigenous bacterial populations has been little analyzed; however, the above-mentioned deleterious compounds may have a profound impact on natural microbial populations, including the selection of antibiotic-resistant variants (11), increased recombination, and, in general, qualitative changes (388) in the microbial species and clones that colonize different habitats. Bioremediation of contaminated environments may help to reduce the potential effect of pollution on microbial ecosystems (11).

Number of contacts between microbes from infected and noninfected hosts (human and animal) and between infected people and the environment. The demographic structure of the human population (338) increasingly facilitates urban crowding, favoring host-to-host contact and the dissemination of infectious diseases among the population.

Global environmental changes. Global environmental changes, including increases in warming and UV exposure, altering the biogeographical limits of bacterial species and thus mixing them with new hosts might serve to accelerate bacterial evolution and increase the number of diseases with previous geographic restrictions. For instance, global warming might increase the prevalence of tropical infectious diseases in areas such as the United States and the European Union.

Human sociological movements. Human sociological movements, including commercial interchanges (330), emigration, travel, war, and establishment of underdeveloped social islands in developed societies might increase contact between people infected with pathogens with a previous closely restricted geographical allocation (see the example of Yanomami Indians in reference 6).

Increase in susceptible hosts. The increase in the number of elderly people, immunosuppressed patients, and children, particularly if they are densely clustered (day care centers, nursing homes), will increase the role of opportunistic pathogens in infectious diseases.

New technologies. New aggressive medical technologies for diagnosis and therapy (not anti-infectious) might reduce the incidence of pathologies such as cancer and diseases of the elderly at the cost of increasing the problem of infections due to opportunistic pathogens (42, 378).

Intensive farming. Intensive farming, which reduces the diversity of animals (the same animal breeds are used in different farms worldwide), might also reduce the diversity of microorganisms colonizing such animals, producing an ecological alteration in the populations of microbes that can come into contact with humans (94). Also, the use of antibiotics for farming purposes might increase the emergence or spread of antibiotic resistance (301, 417).

Viral and virus-like diseases. Viral and virus-like diseases affecting humans and animals, such as AIDS, might evolve and alter the host response against infections.

New antimicrobial strategies. The discovery and use of new antimicrobial strategies, including antibiotics, antivirals, conventional vaccines, pro- and prebiotics, antipathogenic therapies, and modulators of biological response modifiers in the infected host, may be useful for fighting infectious diseases.

Vaccination. New technologies in vaccination (in some cases food-associated; see below) and gene or sequence therapy may be useful for reducing susceptibility to some infections (309).

Bioterrorism. Eventually, bioterrorism will affect antibiotic resistance and virulence, with dangerous and unpredictable consequences (190).

Interestingly, all these factors will also influence antibiotic resistance. Over the last two decades, resistance to antibiotics has crossed the limits of the patient and hospital to become a global environmental phenomenon in which infectious diseases of wildlife and domestic animals might have a deep impact on the emergence and further spread of new human pathogens (94). Everything that promotes bacterial spread among different natural ecosystems and among different hosts (protozoa, animals, plants, and humans) will also potentially affect the spread of resistance. A common theme resulting from all these factors is the possible progressive reduction in bacterial diversity by alteration of the complexity of current ecosystems (see above).

In this regard, intensive farming procedures may have consequences for the future evolution of virulence and resistance. In fact, farming procedures reduce microbial diversity (388). The collapse in the diversity of animal and plant natural variants (races) within agronomically relevant species has already started, because only those variants with high productivity rates are being used worldwide. In fact, most crops are obtained from the seeds produced by a few companies. The result of animal cloning procedures, by which the offspring will be genetically identical to the progenitor, will produce an even larger reduction in the biodiversity of farming animals.

Genetic diversity among hosts is probably a natural barrier to infections, since multiple-passage experiments have shown that adaptation to a new host frequently involves attenuation for the former one (114). From this perspective, a reduction in host diversity will allow bacterial organisms infecting a particular host to spread with greater efficiency to other closely related hosts (identical in the case of clonal farming), eventually increasing virulence. The effect of a possible change of host (for instance, from animals to humans) may result in bacterial attenuation for the former animal host with an unexpected increase in virulence for the new human host (114). The economic and health consequences of these possible changes in bacterial transmission have not been evaluated. The possible outcome is an increase in certain bacterial populations that will alter their potential virulence as a result of interactions with new hosts and then become targets for antibiotic intervention.

If antibiotics are used over the next 50 years as they have been used in the previous 50 years, there is no doubt that the number of replicons and genetic elements carrying antibiotic resistance genes will increase continuously on Earth, and the cost of harboring these genes and vectors will probably be minimized (214, 285), particularly in the more common bacterial populations, thus ensuring their perpetuation in the microbial world.

Strategies against Virulence May Reduce Antibiotic Resistance

Antivirulence strategies tend to reduce the number of bacterial pathogens and may act on the expression of pathogenic mechanisms. In some cases, if the pathogenic mechanism is essential for the lifestyle of the bacteria, the elimination of the pathogenic factor will reduce the overall presence of the pathogen. For instance, nonsystemic toxins, such as necrotic toxins and hemolysins, contribute to the successful growth of the bacterial pathogen inside the host, so that antitoxin vaccination will not only reduce the toxic effect of the toxin, but also contribute to eradicating the bacterial species. In this regard, strategies against virulence may reduce antibiotic resistance, because lower number of pathogenic bacteria implies less antibiotic exposure, a smaller number of antibiotic-resistant mutants, and fewer possibilities of acquisition of antibiotic resistance genes from other bacterial pathogens.

Reducing host-to-host transmission: hygiene and vaccination. Any strategy that leads to a reduction in the number of infections in the host population will reduce the probability of emergence of antibiotic resistance for two reasons: a reduction in the number of bacteria under antibiotic selective pressure, and a reduction in the number of selective environments (different hosts). This results in a decreased probability of selecting different antibiotic-resistant mutants (32).

In this regard, hygiene and vaccination are among the best systems for avoiding infections and resistance. The use of conjugate vaccines against those *S. pneumoniae* serotypes that are more frequently resistant to antibiotics have significantly decreased the rate of penicillin resistance in closed communities (91, 92). As predicted by mathematical models, this decrease is related to the selection of nontargeted and poorly targeted serotypes (219, 220). Nevertheless, in some instances, the more susceptible serotypes may also be the more invasive (163). In this case, if these serotypes are not included in the vaccine, vaccination could increase pathogenicity. Vaccination against *Haemophilus influenzae* type b reduces resistance is this species, as resistance is found more frequently in this serotype (175). In this particular case, both pathogenicity and resistance have dropped simultaneously, as no clear replacement by other types has been documented to date.

In general, the vaccine-mediated reduction in the number of pathogenic organisms and infected hosts reduces the likelihood that resistance will evolve in these bacteria, as acquisition of resistance by mutation and horizontal transfer is a density-dependent phenomenon (see above). Obviously, the principle applies not only to human but also to animal infections and may have an important impact on the development of resistance in the food industry, where antibiotics are frequently used to prevent infections.

In the next few years, we will probably face an explosion in the number of potentially effective novel vaccines. Such a trend is based on the growing use of genomic information together with in silico prediction of useful antigens and massive production of recombinant proteins to be used for immunization of animal models (303). This has been called reverse vaccinology (317) and will doubtless offer a great number of antigenic candidates for the development of a new generation of vaccines. We must insist, however, that when vaccines are developed, the local microbial ecology should be considered.

We have mentioned that antibiotic-susceptible but more virulent bacterial types may emerge because a vaccine is directed against resistant organisms. But it is also true that some vaccines may eventually be more effective in eliminating some antibiotic-susceptible types and therefore select for antibiotic

resistance. This possibility should be considered when a vaccine is introduced into a given human habitat (163, 220). A more optimistic perspective is to consider that prolonged use of antibiotics has reduced the diversity of some bacterial pathogens, so that some pathogenic species may be less able to adapt to novel challenges. If an effective vaccine is available for reducing the number of antibiotic-resistant clones, some pathogens in some parts of the world may become locally extinct (see below).

Antivirulence-directed therapeutic approaches. Antivirulence is considered here as interventions directed against virulent organisms and/or the expression of the virulence determinants. One of the best-known side effects of antibiotic treatment is the replacement of the antibiotic-susceptible commensal flora with resistant organisms. The release of antibiotics in the environment, as well as the use of antibiotics as growth promoters in animal feed, may have a profound impact on bacterial populations. One theoretical possibility to avoid this side effect is to develop antibiotics directed exclusively against virulent organisms. A "magic bullet" is one that only kills virulent bacteria in such a way as to preserve the antibiotic-susceptible phenotype of nonpathogenic bacteria. In this way, commensal bacteria will remain a colonization barrier against infection and will not acquire antibiotic resistance genes. Acquisition of antibiotic resistance genes by nonpathogenic bacteria is detrimental for two reasons. First, these bacteria will constitute a reservoir of antibiotic resistance genes (and antibiotic resistance vectors) that may be transferred to virulent bacteria. Second, antibiotic-resistant bacteria can protect the susceptible ones (eventually pathogenic) from the action of antibiotics (see above).

Although this magic bullet will obviously be desirable, it is not easy to find a lethal target present only in virulent bacteria. Some types of antibiotics act and have been designed in a way that antibiotic resistance will automatically reduce bacterial fitness and consequently the pathogenicity of the resistant organism. For instance, naturally occurring albomycins (124), metalloporphyrins (371), and synthetic derivatives of Fe^{3+} siderophores with antibacterial activity (301) are taken up across the bacterial outer membrane by transport systems for Fe^{3+} siderophores and heme transport systems. Indeed, some antibiotics have been developed that include a siderophore moiety linked to the antibiotic molecule (65, 111, 265). Those antibiotics make use of the siderophore transport system for entry into bacteria. Resistant bacteria can arise as a consequence of mutations in this transport system. However, loss of the iron transport system is detrimental for bacterial growth at the site of infection, so that treatment with this type of antibiotic may severely affect bacterial survival, either by the antibiotic effect itself and by allowing host defenses to cope with the infection due to a mechanism of resistance that has an excessive biological cost for the bacteria.

Vaccination directed against virulence determinants has also been proposed as an effective antivirulence approach to prevention of infectious diseases. Such as all strategies not based on antibiotic treatment, this may help reduce the emergence and spread of antibiotic-resistant bacteria. One example of this strategy is the development of novel vaccines against *S. aureus*. In this organism, the expression of virulence factors is triggered (25) by the RNA III activating peptide (RAP). This peptide is continuously secreted by bacteria, and its activity resembles some other quorum-sensing signals which are also inducers of the expression of virulence determinants (37, 189). Vaccination with RAP protects mice from *S. aureus* pathology (24). This indicates that anti-quorum-sensing strategies might be useful to fight infection.

A different approach for antivirulence vaccination was used by Mason et al. (250). A transgenic potato containing the gene for *E. coli* heat-labile enterotoxin was obtained (250, 379) and used to feed mice. Feeding with such potatoes produced higher levels of antitoxin antibodies than the whole-bacteria vaccine. Thus, this opens the possibility of developing transgenic plants containing several virulence factors that could be used as multisubunit edible vaccines (249).

Strategies Directed against Resistance May Reduce Bacterial Virulence

It has been suggested that the search for and further use of antibiotics active only against antibiotic-resistant bacteria might reverse the problem of antibiotic resistance. The hypothesis of antibiotics able to select susceptible organisms was suggested some years ago (27). For instance, to fight the spread of beta-lactamase-producing bacteria, it may be possible to develop an antibiotic drug whose activation depends on the breakage of a beta-lactam ring. Because only beta-lactamase-positive resistant bacteria would be susceptible to such an antibiotic, beta-lactam-susceptible organisms will be selected. This and other approaches are currently under investigation. Obviously, any new antibiotic able to eliminate (because of the absence of cross-drug resistance) resistant organisms to other drugs has at least the possibility of eradicating resistance. This effect is only possible if the resistant organisms are in a minority with respect to susceptible ones, so that recolonization after therapy will occur from within the host and from other hosts with susceptible bacteria.

However, until now, the best (and maybe only) strategy against resistance is a good policy for the prescription and use of antibiotics (169). Rotation in antibiotic use has been suggested (322) and even implemented, in some cases, with an improvement in antibiotic resistance rates (282). However, the levels of resistance never drop to zero, so that rotation of antibiotics is useful only transiently (332). Antibiotics must be used only when needed (215, 223), and the consumption of antibiotics for purposes other than the treatment of infectious diseases must be avoided as much as possible. For instance, the ban on the use of avoparcin in animal feed has curbed the development of vancomycin resistance in the European Union (23). It has been discussed whether the ban would be followed by a reduction in the productivity and an increase in the morbidity and mortality of the animals as a consequence of an increasing number of infections (23, 115). However, neither a reduction in productivity nor an increase in the number of infections has been observed after discontinuing the use of antimicrobial growth promoters (115), probably because hygienic measures were implemented at the same time as the ban (23).

Strategies against the emergence and further spread of antibiotic resistance genes in bacterial populations will obviously reduce virulence because infections will be easily treated and

the disease will disappear. We cannot forget that during the 1980s, it was common to believe that infections would not be a relevant clinical problem in the future of mankind. However, it became clear in the 1990s that this was not the case. Some infections became untreatable by conventional chemotherapy, and this produced a reemergence of the problem of infectious diseases (378). This renaissance has not been a consequence of the introduction of novel bacteria with a higher virulence potential, but mainly as a result of emergence of the same families of bacteria which now are resistant to antibiotics. Because antibiotics are the very last line of defense incorporated along human evolution in its fight against infections, breaking down this defense will restore the problem of infectious diseases to the situation of the preantibiotic era. Therefore, antiresistance strategies will clearly diminish the overall virulence of bacterial pathogens, because bacteria will remain susceptible to treatment so that they will not produce disease. To achieve this goal, novel antibiotics are required. (419)

Drug diversification. Although several antibiotics are currently being used in therapy, all belong to a few structural families, so that resistance to one antibiotic frequently produces resistance to other members belonging to the same family. Novel antibiotics belonging to different structural families than those currently in use are then needed in order to fight against antibiotic-resistant bacteria

Two approaches can be combined in the search for novel antibiotics. One is the classical search for novel inhibitors of lethal bacterial targets either by using natural sources or by screening of synthetic compounds. The other is the search for inhibitors of resistance systems, with the aim of recovering a susceptible phenotype in a previously resistant population. Examples of the latter strategy are the beta-lactamase inhibitors (236) currently used in clinical practice, and the inhibitors of MDR pumps which are under development by some pharmaceutical companies.

Concerning the search of inhibitors of classical targets, major efforts are currently under way in order to use the knowledge derived from the sequencing of whole microbial genomes (325). The advantages derived from the use of combinatorial chemistry (109) and rational design by computerized molecular modeling of crystal structures of potential targets (331), the use of new methods for the screening of natural compounds (69), and the increasingly refined methods of high-throughput screening (64, 87) will be useful for developing novel families of antibiotics in the future. Nevertheless, we must still wait for the introduction of new antibiotics developed by using these methodologies.

In the meantime, new fluoroquinolones such as moxifloxacin (26), gemifloxacin (229), and gatifloxacin (298), new tetracyclines such as glycylcycline (375), streptogramins such as the combination quinupristin/dalfopristin (200), macrolide-related agents such as ketolides (107), and a new family of antimicrobials, oxazolidinones (110), have been launched and/or in the last phases of development. Some new families and resistance inhibitors are under development. These include the novel inhibitors of class C beta-lactamases such as boronic acids (411) and inhibitors of MDR efflux pumps (227, 319, 369)

The diversity of clones within a given bacterial species is severely reduced by antibiotics. In some instances, the species may maintain a certain degree of diversity when the resistance determinant disseminates by horizontal transfer into different clones, and if a mechanism of diversification is available, for instance, capsular transformation in *S. pneumoniae*. Nevertheless, even in the case of species able to exchange DNA, a progressive simplification of the population structure is expected to occur. In *S. pneumoniae*, prolonged challenge with beta-lactams has selected a relatively small number of "international resistant clones," certainly at the expense of reducing the number of susceptible clones. It has been discussed that this reduction in diversity may lead to the potential extinction of some highly virulent but antibiotic-susceptible clones. In this sense, the overuse of antibiotics may have reduced the overall pathogenicity of certain organisms. In any case, the antibiotic-driven reduction in diversity of the bacterial clones may cause extinction of some of these bacterial pathogens, using either vaccines against the reduced number of resistant clones or new antibiotics to which the resistant bacteria are susceptible, or both strategies simultaneously. Implementation of barriers to transmission will increase the possibility of reaching extinction. It seems that a low-diversity population structure will reduce the possibility of maintaining a pathogen under these new pressures.

INFLUENCE ON EVOLUTION OF THE HOST

The sentence "I am myself and my circumstances" from the Spanish philosopher Ortega means, in biological terms, that not only genetics but also the environment is relevant to phenotype. In the case of humans, it might also mean that the human body is formed by 10% mammalian cells and 90% prokaryotic cells (our normal microbiota) (41), each of which can influence the evolution of the other. The human host is also surrounded by a plethora of environmental microorganisms, some of which may interact transiently with the human body. Therefore, it is clear that the ability of bacteria to grow and damage human tissues has most probably had an effect on human evolution. For instance, the build-up of the extremely complex system of immunological responses involved in the defense against infection that was selected during the evolution of animals was necessary (and, so, selectable) only because of the existence of virulent microorganisms.

In this regard, it has been suggested that infectious diseases might be the major evolutionary force driving the high diversity displayed by the major histocompatibility complex (178). Body temperature, which is higher than the mean environmental temperature, limits the growth of microorganisms in humans to those that can grow in a narrow range around 37°C. In fact, it has been demonstrated that pathological hypothermia favors infection by environmental bacteria such as *Pseudomonas putida* (76). We thus speculate that body temperature is a very first line of defense that precludes infection by environmental microorganisms. It is thus possible that this anti-infective function together with several other selective pressures has contributed to select 37°C (a temperature that requires that constant metabolic activity be maintained) as the human body temperature. These examples illustrate that virulent microorganisms might have had a relevant effect on host evolution, as suggested by Haldane more than 50 years ago (159).

Since microorganisms can overcome human anti-infective defenses and kill the human host, infection might select the

most resistant (in terms of infection) genetic backgrounds in the host population (309). In such a way, the prevalence of specific infectious microorganisms in different geographic allocations might be a driving force in human evolution. A very well known example of this viewpoint is the prevalence of sickle cell anemia in regions with a high rate of malaria infections (198). Sickle-shaped hemoglobin is protective against malaria. Thus, infection by *Plasmodium falciparum* has produced a bias in the number of sickle cell heterozygotes. The percentage of sickle cell homozygous individuals did not show such a relevant increase because these people have a high mortality risk caused by complications of sickle cell disease.

A more recent example is the prevalence of cystic fibrosis in Caucasian populations, which is higher than predicted by genetic analysis. Cystic fibrosis is a genetic disease caused by the lack of activity of a chlorine channel (CFTR). CFTR is also the receptor for some bacterial pathogens, such as *Salmonella enterica* serovar Typhi. Therefore, it has been suggested that cystic fibrosis could prevent infections by this bacterial species (302). In such instances, an increased risk of infection by *S. enterica* serovar Typhi might select populations with a bias in the percentage of cystic fibrosis patients.

In a similar way, it has been found that resistance and susceptibility to virulent *M. tuberculosis* is a complex genetic trait (195), so that different genetic backgrounds make people more and less susceptible to developing clinical tuberculosis once they have been infected with *M. tuberculosis*. It has been described that Yanomami Indians of the Brazilian Amazon have diminished cell-mediated immune responses against *M. tuberculosis* compared with control individuals of European extraction living within the same region. Yanomami Indians remained isolated until the mid-1960s and have not been in contact with *M. tuberculosis* until recently. As stated by the authors of that work, *M. tuberculosis* infections exerted a powerful selective pressure, "resulting in the elimination of a significant proportion of highly susceptible individuals over their reproductive age" (362). This selection occurred before the availability of antituberculosis treatment in the 1950s and might be occurring now if the treatment is not effective due to the emergence of antibiotic-resistant *M. tuberculosis*.

The same occurs for other infectious diseases. We must to keep in mind that the contact between Europeans and native populations in America produced dramatic effects on the Native Americans due to the dissemination of "European" infectious diseases among them. Whereas Europeans had been in close contact with these infectious agents, with a resulting selection against the most susceptible individuals occurring a long time ago, the first encounter between the native population and the European "alien" infectious agents caused the death of a great number of people. It has also been suggested that infections in the New World have been an important force for selecting the mixed race (mestizo) produced by the mating between Spaniards and Indians. As suggested by the authors, mestizos should be immunologically more capable of defending themselves against the different infectious agents brought over from the Old World (274) and maintaining their lower susceptibility to New World infections, so that they should be more adapted than Europeans and Native Americans to the new conditions produced by the introduction of novel pathogens in the environment.

These examples illustrate that infections have been important driving forces in human evolution. We have to keep in mind that several genetic changes might have been selected over human evolution just because they are protective against infections. However, we have only detected those changes which, like cystic fibrosis and sickle cell disease, are associated with a relevant genetic disorder. More recently, the study of infection-resistant phenotypes by classic human genetic analysis (309) is shedding new light on the potential role of infectious diseases as driving forces in host evolution. The use of antibiotics and the development of antibiotic resistance mechanisms might also have an effect on host evolution, because they will change the risk for infection.

To illustrate, we can speculate that the introduction of effective treatments (antibiotics, prophylaxis, vaccines) against *S. enterica* serovar Typhi might eventually reduce (in the long term) the prevalence of cystic fibrosis to the percentage of population predicted by purely genetic criteria. Also, as stated previously, effective antituberculosis treatment will allow the highly susceptible population to survive, because *M. tuberculosis* would not exert any selective pressure. Antibiotic treatment will then have the effect of removing selective pressure due to infections, and antibiotic resistance will have the effect of returning such selective pressure.

We want to stress here that, in any case, the time scale for a detectable effect of antibiotic treatment on human evolution is a lot longer than the time scale needed for bacterial evolution due to the extremely different lifetimes of the two types of organisms (from minutes to hours for bacteria, several decades for humans), so that a direct effect of antibiotics in human evolution is unsuitable unless antibiotics are maintained as the main choice for treatment of infections over several centuries. However, the most rapid and evident effect of antibiotic treatment will be on the composition of normal microflora because antibiotic treatment will select for resistant variants not only in the treated virulent microorganisms, but in the commensal microflora also (see below).

Changes in Normal Host Microbiota

Might virulent and/or antibiotic-resistant bacteria change host microflora? It is clear that a bacterial pathogen needs to successfully compete with normal host microflora in order to produce an infection, so that a temporal displacement of commensal bacteria might occur over the very first steps of infection. On the other hand, virulent bacteria might either select "resistant" hosts (see above), in which they can multiply without producing any relevant disease, and attenuated bacterial variants might also be selected. In any of these cases, the previously virulent bacteria might finally be incorporated as a commensal microorganism into the normal host microflora. Antibiotic treatment will also displace normal host microflora, allowing the recolonization by exogenous antibiotic-resistant bacteria as well as by antibiotic-resistant mutants belonging to normal microflora. This effect will probably be buffered in the long run, because human populations are not always under antibiotic treatment, so that the risk for selection of resistant variants will be lower for commensal microflora than for virulent microorganisms (see above).

Nevertheless, it cannot be ruled out that because of the huge

consumption of antimicrobials in the world, some bacterial members which form the normal host microbiota will be replaced by others. For instance, highly sporulating gram-positive bacilli may replace (because spores are antibiotic resistant) less-sporulating organisms. Some *Lactobacillus* species are intrinsically less resistant to antibiotics than others and will be progressively selected. In general, bacteria with lower growth rates (less susceptible to most antibiotics) may also be selected. It can be predicted that those changes will produce effects, for the moment largely unknown, on human health.

Even for the same bacterial species, a replacement of human-adapted strains by animal-adapted strains might occur. For instance, natural populations of *E. coli* are organized in an ecotypic structure where adaptation to the host plays an important role (363). As discussed before, the use of antimicrobials as growth promoters selects antibiotic-resistant clones in the commensal microbial flora of farming animals (394, 418). Those clones that are not good colonizers of humans might replace human-adapted antibiotic-susceptible clones in the case of antibiotic treatment, thereby breaking down the close association between bacterial clones and their host. It is true that current bacterial diversity will probably offer "almost equivalent" resistant organisms to replace those that may be under danger of extinction. Nevertheless, most probably our intestinal flora will change, and therefore our microbial heritage for the future of humankind (the "historical host" in the future) will be adulterated. Again, the evolutionary consequences are totally unknown.

Microbial evolution is not a reversible process. The release of huge quantities of antibiotics on the Earth, together with other environmental toxic compounds, and the changes in the population structures of human (338) and animal hosts have profoundly changed the interactions of microbes and humans. The possibility of wide restoration of a wild antibiotic-susceptible bacterial flora in nature is beyond our current abilities.

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