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MINIREVIEW

The Search for Antimicrobial Agents Effective against Bacteria Resistant to Multiple Antibiotics

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INTRODUCTION

The discovery, development, and clinical use of antibiotics during the 20th century have decreased substantially the morbidity and mortality from bacterial infections. The antibiotic era began with the therapeutic application of sulfonamide drugs in the 1930s, followed by a "golden" period of discovery from approximately 1945 to 1970, when a number of structurally diverse, highly effective agents were discovered and developed (16). However, since the 1980s the introduction of new agents for clinical use has declined, reflecting both the challenge of identifying new drug classes and a declining commitment to antibacterial drug discovery by the pharmaceutical industry (11, 42, 53, 63). The same period with a reduced rate of introduction of new agents has been accompanied by an alarming increase in bacterial resistance to existing agents, resulting in the emergence of a serious threat to global public health (7, 9, 28, 39, 49, 60, 63, 64).

The resistance problem demands that a renewed effort be made to seek antibacterial agents effective against pathogenic bacteria resistant to current antibiotics. This minireview reviews the status of research in this critical therapeutic area. We reevaluate the potential of older, unexploited agents and review current approaches to the discovery of new agents, including the identification of new molecular targets for antibiotic action. Although other approaches such as the use of vaccines, monoclonal antibodies, hematopoiesis-stimulating factors, and various immunoregulatory cytokines may prove to have utility against infections caused by antibiotic-resistant bacteria (7), this minireview is limited to discussion of antibacterial agents and strategies for the detection of new molecular targets.

REEVALUATION OF OLDER, UNEXPLOITED AGENTS

As noted by Zahner and Fiedler (72), the discovery and successful development of the antibiotics currently used for chemotherapy led to termination of interest in other antibiotic classes discovered during the 1960s and 1970s. These underexploited agents include the everninomycins, avilamycins, kirromycins, lankacidins, sideromycins, pleuromutilins, and indolymycin, as well as several antimicrobial peptides (72). A number of these agents appear to have structures and modes of action that are distinct from those of the antibiotics in current use, suggesting that cross-resistance with agents already in use may be minimal (72). Therefore, reevaluation of these older agents with a view to developing them for use against organisms resistant to current agents may be worthwhile (72). Nevertheless, the development of the avilamycins and pleuromutilins for human use could be contraindicated because they have already been utilized for animal husbandry and veterinary medicine (12, 44, 72). Use of these agents in animals may have led to the selection of resistant environmental isolates that could serve as a source of resistance determinants in human pathogens, thereby potentially compromising the therapeutic potential of new avilamycin or pleuromutilin analogs in humans.

Treatment of infections with bacteriophages also constitutes an older therapeutic concept that has not been developed (56). Effective bacteriophage therapy has been demonstrated in certain experimental infections (56), and in contrast to antibacterial drugs, benefits might result from continued production of the antibacterial agent at the site of infection due to the replicative nature of bacteriophage infection. The potential antigenicity of bacteriophages, their narrow spectrum of activity, and their ready development of resistance by loss of receptors probably explain why they have not been developed as therapeutic agents.

RATIONAL APPROACHES TO DRUG DESIGN BASED ON UNDERSTANDING THE BIOCHEMICAL BASIS OF BACTERIAL RESISTANCE

The biochemical basis of acquired resistance to most classes of antibiotics is now known (6, 10, 29, 39, 49, 52, 61), and elucidation of these mechanisms has been important in designing rational strategies that can be used to counteract resistance. Examples of the various approaches are considered below, focusing where appropriate on the newer, investigational agents arising from application of this rational strategy.

Analogs of existing antibiotics stable to enzymatic inactivation. Enzymatic inactivation of antibiotics is an important mechanism of bacterial resistance to β-lactam antibiotics, chloramphenicol, aminoglycosides, and macrolides (10, 39, 49, 52, 61). To counter this problem, enzyme-stable analogs have been developed and marketed, most notably with the β-lactams, e.g., the isoxazolyl penicillins (26, 40, 49, 50), imipenem and meropenem (26, 37, 40, 49, 71), and the aminoglycosides, e.g., amikacin (26). In other cases analogs stable to enzymatic inactivation have been discovered, e.g., 3-fluoro-3-deoxy derivatives of chloramphenicol (52, 61), but they have not been developed because of potential toxicity or poor pharmacokinetic properties. If there is an increase in the prevalence of carbapenemases in clinical isolates (30), then the synthesis of new carbapenem analogs stable to these emerging β-lactama-

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ses may become a focus for future research activities in the pharmaceutical industry.

Inhibition of bacterial enzymes that inactivate antibiotics. The bacterial enzymes that degrade or modify antibiotics are themselves potential targets for drug action, leading to combination products which contain an antibiotic and a specific inhibitor which protects the antibiotic from enzymatic inactivation.

The concept of using enzyme inhibitors in combination with antibiotics to block bacterial degradation of the antibiotic has been applied successfully to a number of established penicillins (10, 26, 49). Clavulanic acid was the prototype molecule that established the value of this strategy (48). It exhibits weak antibacterial activity, but binds with high affinity and essentially irreversibly to many bacterial β-lactamases. Clavulanic acid thus protects β-lactam antibiotics from destruction and is available commercially in combination with amoxycillin (Augmentin) and ticarcillin (Timentin). Other β-lactamase inhibitors have also been introduced more recently (10, 49), e.g., sulbactam, marketed in combination with ampicillin (Unasyn), and tazobactam, marketed in combination with piperacillin (Tazacin and Zocin).

Although enzyme inhibitors have been successful in combating microbial β-lactamases, application of this principle to the inhibition of other antibiotic-inactivating enzymes has not led to the identification of any candidates for development (10, 61).

Analogs of existing antibiotics not recognized by bacterial efflux pumps. In some isolates resistance to the macrolides, quinolones, and tetracyclines is mediated by efflux pumps that prevent antibiotic accumulation by the bacterium (6, 10, 29, 39, 49). The synthesis of analogs of these antibiotic classes that are not recognized by efflux pumps, yet that retain antibacterial activity, provides in principle a solution that can be used to circumvent transport-mediated resistance. The utility of this approach is illustrated by the recent discovery of the glycylcyclines, a new class of tetracycline analogs (62). These analogs exhibit antibacterial activity against organisms expressing efflux-based resistance to older tetracyclines which is attributable to failure of the efflux proteins to recognize and export the new analogs (55, 62). With the exception of resistance to tetracyclines, efflux is not a common mechanism of resistance to other agents in clinical isolates (6, 29). Nevertheless, development of macrolides and quinolones as well as tetracyclines not recognized by efflux systems may be beneficial in view of concerns that efflux-based antibiotic resistance mechanisms may become more prevalent (6).

Inhibition of efflux pumps that remove antibiotics from the cell. The efflux systems that remove antibiotics from bacteria are themselves potential targets for drug action that would lead to products containing an antibiotic and an inhibitor which prevents efflux of the drug from the cell. Bacterial efflux pump inhibitors have been discovered, but their properties as revealed to date are not sufficiently attractive to warrant development (10). Further efforts in this area are to be encouraged in view of the emergence of efflux-based resistance in clinical isolates (6, 29).

Analogs of existing antibiotics that bind to modified target sites in resistant bacteria. Modification of the target site for antibiotic action results in resistance to a number of antibiotics, including the tetracyclines, β-lactams, and glycopeptides (49). The chemical synthesis of analogs of these antibiotic classes has yielded new derivatives that bind to the refractory targets.

The glycylcyclines described above inhibit protein synthesis on ribosomes that express resistance to older tetracyclines via target site protection at the ribosome (47, 62). The new carbapenem analogs L-695,256 and SM-17466 have improved affinity for the *mecA* gene product of methicillin-resistant staphylococci (35, 58). The *mecA* gene encodes a unique penicillin-binding protein (designated PBP 2′) which, through a low affinity for β-lactam antibiotics, including imipenem, confers resistance to these agents (39, 49). Finally, *N*-alkyl-substituted glycopeptides represent important leads in the generation of new glycopeptide antibiotics with activity against vancomycin- and teicoplanin-resistant gram-positive bacteria (41). These organisms possess modified peptidoglycan structures refractory to the binding of the older glycopeptides (2, 49).

IDENTIFICATION OF NEW BACTERIAL TARGETS AND NEW ANTIMICROBIAL AGENTS

The examples described in the previous section represent rational approaches to circumventing resistance to classical antibiotics. This strategy depends principally on the synthesis of repertoires of new analogs related to known antibiotics to create structural modifications that thwart the resistance phenotype.

An alternative approach to the problem of emerging resistance to current antibiotics is to seek structurally novel antibiotics that inhibit new molecular targets. Such agents are unlikely to be susceptible to existing mechanisms of resistance because of their structural novelty and unique mode of action. New molecular targets for antibiotic action are discussed in the following sections.

New molecular targets involved in bacterial growth. Nucleic acid, protein, and cell wall syntheses are already the targets of existing antibacterial agents (16, 49). Nevertheless, the complexities of these processes provide a rationale for continuing to screen for inhibitors acting on new molecular targets within these pathways. Indeed, this position is supported by the discovery and current development of the oxazolidinones (23, 24, 57), which inhibit bacterial protein synthesis by a novel mechanism (54). The antibiotic mupirocin inhibits bacterial isoleucyl-tRNA synthetase (20), which charges its cognate tRNA species with isoleucine. Mupirocin inhibits the formation of isoleucyl adenylate, the first step of this aminoacylation reaction, leading to depletion of charged $tRNA^{Ile}$. The resulting amino acid starvation not only leads to inhibition of protein synthesis but also has widespread effects on cellular metabolism through induction of the stringent response (21, 69). Apart from isoleucyl-tRNA synthetase, bacteria contain additional aminoacyl tRNA synthetases required for ligation of other amino acids to tRNAs (36). Therefore, these essential enzymes are attractive targets for new antibacterial agents, and differences in the structure of aminoacyl-tRNA synthetases from prokaryotes and eukaryotes (36) may be sufficient for the development of further selective antibacterial drugs. The products of the recently identified *fem* genes involved in the synthesis of the pentaglycine interpeptide bridge in the staphylococcal cell wall may also constitute appropriate molecular targets for new antistaphylococcal agents (27).

In the search for new microbial targets, a number of other processes that would affect bacterial growth have been suggested. These include inhibition of lipoprotein and lipopolysaccharide biosynthesis in gram-negative bacteria, cell division, and metabolite transport (1, 59). Many of these pathways have been validated as candidate targets for antibiotic action by showing that mutations in the structural genes encoding the pathways are lethal (59). Agents that inhibit steps in some of these pathways have been identified, e.g., inhibitors of lipopolysaccharide biosynthesis (18). Unfortunately, work with

TABLE 1. IVET systems and the *ivi* genes that they have revealed

System	Species	In vivo selection	Examples of *ivi* genes	Reference
pIVET1	*S. typhimurium*	Purine auxotrophy	Antisense transcript that may down-regulate lipopolysaccharide synthesis; novel, unknown genes	33
pSR37	*L. pneumophila*	Thymine auxotrophy	Oxidative stress genes; novel, unknown genes	46
pIVET8	*S. typhimurium*	Resistance to chloramphenicol	Fatty acid degradative enzyme: possible protective mechanism against toxic host metabolites	34

these agents does not appear to have progressed beyond the investigational stage, since no antibacterial agents with targets in these essential pathways are in development. This probably reflects a number of factors. For example, it may be difficult to devise an adequate biochemical assay for screening of inhibitor candidates, and in other cases, even though an inhibitor is discovered, it may not exhibit activity against intact organisms due to exclusion by the bacterial cell envelope. Furthermore, even though an investigational drug has adequate antibacterial activity in vitro, it may not be possible to make progress with the compound if it has poor pharmacokinetic properties or is metabolically unstable.

New molecular targets involved in bacterial infection. Recent interest in the mechanisms by which pathogenic bacteria cause disease has raised the possibility of designing new agents that act against gene products expressed primarily or exclusively during infection (7). An anticipated advantage of developing such agents will be the likely absence of preexisting resistance mechanisms (7). The search for new microbial targets associated with infection will be substantially assisted by new techniques designed to detect bacterial genes expressed selectively in vivo (discussed below). However, even before the introduction of these techniques, other approaches had already identified a number of gene products that are involved in infection and that offer good candidates for new intervention strategies. These are also discussed below.

(i) Gene products known to have a role in infection as candidates for inhibition by novel antibiotics. Two examples of gene products known to have a role in infection are provided, whereby existing information on pathogenic mechanisms might lead to the discovery of novel antibiotics.

It has been known for some time that assimilation of iron by pathogenic bacteria is essential for their growth in vivo (4). Indeed, bacterial iron assimilation was one of the first infection-related processes to be suggested as a potential target for antibiotic action (3), and sophisticated screening systems have now been devised to detect inhibitors of bacterial iron uptake (65).

Surface-expressed bacterial proteins play a central role in the pathogenicity of many bacteria, particularly in gram-positive species, where they promote bacterial adhesion to host tissues, facilitate subsequent invasion of the tissue, and confer resistance to phagocytosis (13, 17). In gram-positive bacteria many of the surface proteins are attached to the cell wall at the C terminus by a specific anchoring process involving proteolytic cleavage of secreted proteins (38). Inhibition of the anchoring process by an antibiotic should prevent the pathogen from establishing disease or render it susceptible to the host defense system (38).

(ii) Identification by IVET of new genes with a role in infection. In vivo gene expression technology (IVET) is a new technique which distinguishes genes expressed during growth both in vitro and in vivo (so-called housekeeping genes) from those that are expressed selectively during infection in vivo (virulence genes or in vivo-induced [ivi] genes). The IVET approach uses the host to enrich for genes that are expressed in host tissues during the pathogenesis of infection and can be applied, in principle, to any pathogen.

The original IVET experiments were performed with *Salmonella typhimurium* by using a system (pIVET1) based on the in vivo complementation of purine auxotrophy (*purA*) (33). The IVET approach has been extended to *Legionella pneumophila* by using in vivo complementation of thymine (*thy*) auxotrophy (46). IVET techniques that use in vivo complementation of auxotrophic markers may also prove to be applicable to pathogenic mycobacteria (22). For instance, several amino acid auxotrophs of *Mycobacterium bovis* BCG which fail to grow in mice have recently been isolated. These strains might therefore be complemented in vivo by wild-type genes driven by promoters active during infection.

More recently, the original IVET technique (33) has been modified to allow for the in vivo selection of *S. typhimurium* genes based upon expression of resistance to chloramphenicol (34). Intriguingly, this particular approach is based on the identification of promoters active during infection of plants by *Xanthomonas campestris* pathovar *campestris*, the causative agent of crucifer black rot (43). The application of chloramphenicol to plants can inhibit the growth of prokaryotic plant pathogens in vivo, generating conditions for selection of promoters active *in planta* fused to a promoterless gene encoding chloramphenicol acetyltransferase.

In vivo-induced genes in pathogenic bacteria identified by the use of IVET systems are described in Table 1. However, these systems have potential technical limitations which may restrict analysis of gene expression during infection. For example, the *purA* and *thy* systems require that auxotrophy be complemented throughout the course of infection. The selection system employing chloramphenicol resistance is limited by the pharmacodisposition of the drug since effective selection requires that the antibiotic be present in tissues at a concentration sufficient to maintain selective pressure on the test pathogen. An improved screening system that may overcome these limitations is based on in vivo expression of γδ resolvase (5, 32). The γδ resolvase is a site-specific recombinase which catalyzes excision of DNA flanked by direct repeats of *res*, the DNA sequences at which resolvase functions. The basis of the system is a plasmid-borne, promoterless γδ resolvase gene, upstream of which chromosomal fragments are randomly cloned (Fig. 1). The recombinants are then introduced into the animal model for the infection, and the chromosomal fragments containing the promoters that are expressed in vivo drive production of the resolvase. To date, this system has been applied only to a few known promoters in *Vibrio cholerae* (5, 32) in which expression of the resolvase is detected by its action on a cassette borne on the same vector which contains a tetracycline resistance (*Tc-r*) gene flanked by two *res* sites. Cells in which the resolvase is expressed are rendered phenotypically sensitive to tetracycline (*Tc-s*) due to excision of the *Tc-r* gene. By extension, random cloning of promoters into pIVET6 followed by recovery of clones where the resolvase has been

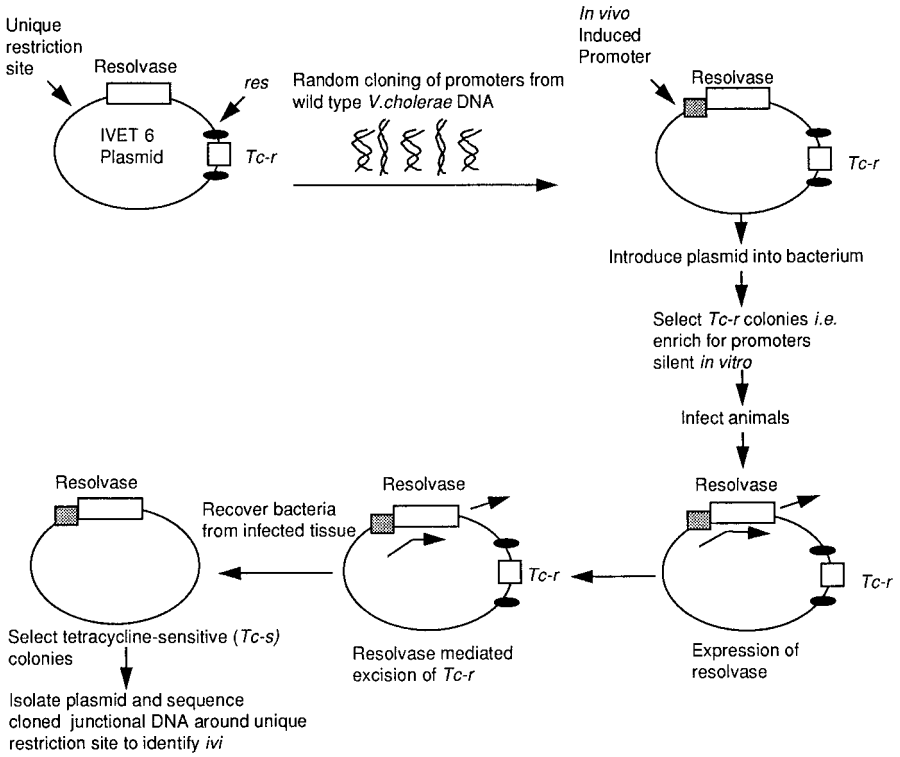

FIG. 1. pIVET6 system (5) and its potential application to the identification of in vivo-expressed genes in *V. cholerae.*

expressed in vivo should permit identification of in vivo expressed structural genes (Fig. 1) (32).

In contrast to the systems described in Table 1, the pIVET6 vector has the attraction that it does not depend upon the maintenance of in vivo selection conditions, which may impose limitations on the identification of in vivo-expressed genes (see above). Furthermore, IVET systems based on the γδ resolvase are particularly suited to establishing the temporal expression of any particular gene expressed in vivo (32). The γδ resolvase system should also permit studies of gene expression in organisms in which auxotrophic mutations and complementation are not possible (34).

(iii) Identification of new genes with a role in infection by analysis of bacterial transcripts expressed in vivo. The foundations of the identification of new genes with a role in infection by analysis of bacterial transcripts expressed in vivo arise from the demonstration that RNA transcripts can be used to detect expression of bacterial genes responding to various stimuli imposed during laboratory culture. Chuang and coworkers (8) identified *Escherichia coli* genes expressed in response to altered culture conditions by preparation (reverse transcription) of total cDNA, followed by DNA dot blot hybridization with an ordered *E. coli* genomic library constructed in phage lambda. The availability of an ordered genomic library for *E. coli* greatly facilitated the identification of genes corresponding to the expressed mRNAs. To validate the system for detection of altered mRNA levels, control experiments involving induction of the lactose operon by addition of isopropyl-β-D-thiogalactopyranoside to laboratory-grown cultures were conducted. Elevated hybridization signals corresponding to clones containing *lac* sequences were indeed detected.

In an alternative approach, applied to the identification of *S. typhimurium* genes expressed in vitro following oxidative stress, arbitrarily primed PCR fingerprinting of differentially expressed RNA was used to detect differences between RNA populations (70). This involved the use of arbitrarily selected primers at low stringency for first- and second-strand cDNA synthesis, followed by PCR amplification of the products (68). The differentially amplified products could then be cloned and sequenced to identify stress-induced genes (68).

A subtractive hybridization approach has been applied to bacteria grown under ex vivo conditions (45, 66) and has been used to enrich for genes specifically expressed during growth of *Mycobacterium avium* (45), *Mycobacterium tuberculosis* (25), and *Haemophilus influenzae* (67) in tissue culture cell lines while eliminating the majority of the housekeeping genes. The final subtractive hybridization products can be sequenced directly by PCR-based protocols or can be used as probes to identify genes in appropriate genomic library clones (66). Quinn and coworkers (25, 66, 67) used total RNA for the subtractive hybridization to remove housekeeping genes (Fig. 2), whereas Plum and Clark-Curtiss (45) subtracted rRNA in a separate first-stage hybridization.

Transcript analysis has not yet been widely applied to the detection of bacterial genes in organisms recovered from experimentally infected animals. However, Chuang et al. (8) have demonstrated the exciting potential of the method by analysis of genes expressed by *E. coli* isolates recovered from a gastrointestinal infection of gnotobiotic mice. Several genes known to be expressed under anaerobic conditions were detected in vivo, as was enhanced expression of genes probably required for protection of bacteria from bile salts in the gastrointestinal tract (8). The latter may represent novel stress-induced efflux systems that decrease the concentration of bile salts in the local environment of the bacterial cell envelope (31).

Identification of in vivo-expressed genes by transcript anal-

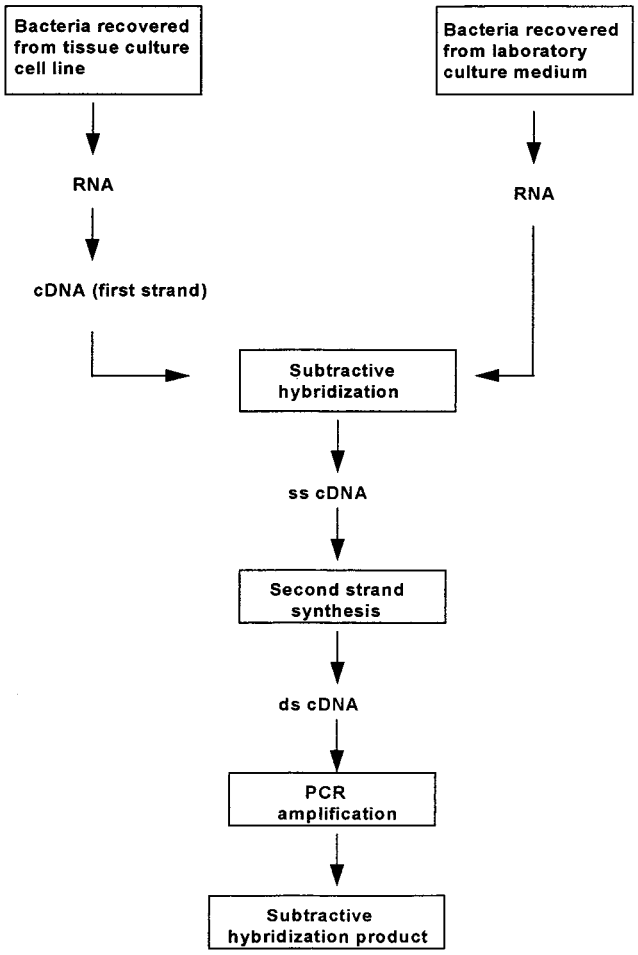

FIG. 2. Outline of the mRNA subtractive hybridization technique (25, 45, 66, 67). ss, single stranded; ds, double stranded.

ysis is likely to be further assisted by techniques developed to monitor gene expression in higher organisms. For example, the microassay technology described by Schena et al. (51), in which *Arabidopsis thaliana* mRNA expression was examined by using fluorescently labelled cDNA probes, could be applied to prokaryotic systems.

(iv) Identification by signature-tagged mutagenesis of new genes with a role in infection. Signature-tagged mutagenesis represents a third approach to the identification of genes required for infection (19). This technique comprises a negative selection procedure whereby transposons containing unique sequence tags are used for mutagenesis to inactivate genes with a role in infection. Inactivation of the virulence genes renders the mutants avirulent in experimental infections, but their capacity to grow in vitro is not impaired. Avirulent mutants are revealed by detection of signature (sequence) tags in organisms present in the inoculum, but not in organisms recovered from experimental infections. So far this technique has only been applied to the analysis of *S. typhimurium*, in which it has resulted in the identification of a number of new virulence genes (19). Application of the technique to a wider range of bacteria is likely to identify a number of new gene products that may be amenable to inhibition by new antibiotics.

CONCLUSIONS

The development of antibiotics for the chemotherapy of bacterial infections represents one of the most remarkable achievements of this century. Unfortunately, the increasing emergence of acquired resistance to antibiotics seriously threatens their effectiveness for the therapy of both nosocomial and community-acquired infections. The development of new prophylactic and therapeutic procedures is urgently required to meet the challenges imposed by the emergence of bacterial resistance.

This minireview has considered a number of possible solutions to the problem, ranging from reevaluation of older agents and continuation of rational approaches to drug discovery to the identification of new molecular targets for antibiotic screening initiatives. Recently, we have argued in favor of a strong commitment to the targeting of virulence genes to create new antibiotics, believing that agents which interfere with infection may be less susceptible to the emergence of resistance than current agents (7). The opportunities to target infection processes are exciting, and as discussed in this minireview, the search for in vivo-expressed functions as drug targets has been substantially enhanced by the introduction of new techniques such as IVET, RNA transcript analysis, and signature-tagged mutagenesis. The search for new molecular targets associated with infection will also be assisted by large-scale bacterial genome sequencing projects (14, 15).

To date, relatively few virulence or in vivo-induced genes have been reported in the literature (e.g., see Table 1). More information on the nature and role of these novel gene products during infection is required to determine whether they are suitable targets for the development of screening protocols. Furthermore, it can be anticipated that the discovery of additional virulence genes may lead to the identification of new gene products that are more appropriate as targets for antibiotics than those currently reported. Before devising functional biochemical assays for the novel gene products induced during infection, various factors in the final selection of targets for screening will need to be considered. These will relate to their absence from eukaryotic tissues, their location and abundance in the bacterial cell, and the overall range of bacterial species in which they are found.

Targeting of in vivo-expressed functions may lead to narrow-spectrum agents because the targets could be highly specific for each pathogen. Therefore, the use of such drugs will require the development of rapid and accurate technologies for microbial diagnostics (7). In addition to minimizing the selection of resistant isolates, drugs that target infection processes may have the added advantage of not disturbing the normal commensal flora. On the other hand, since new drugs that specifically interfere with infection may not possess intrinsic antibacterial activity, it is likely that such agents will need to work in concert with host defenses to eradicate infection. Therefore, a possible limitation in the use of the new agents could be their lack of efficacy in immunocompromised patients.

New molecular techniques are providing investigators opportunities to discover families of novel antibiotics that specifically target products associated with infection in vivo. Nevertheless, because of the time required to develop and clinically evaluate new agents, it will be several years before any such agent is available for routine clinical use (7). In the meantime, we will be faced with increasing bacterial resistance to current antimicrobial agents.

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