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Applications of genetics to studies of bacterial virulence

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Classical genetic techniques have made it possible in many instances to discern which bacterial factors are directly involved in causation of infection, as opposed to those that are associated with but do not directly contribute to virulence. By use of these methods as well as monoclonal antibodies, recombinant DNA, and other new techniques it has been shown that bacterial virulence is complicated, with involvement of many different bacterial factors at each step of infection; bacterial factors that facilitate one step of infection may actually impede a subsequent step. Interestingly, a large number of genes involved in toxin production or bacterial cell-surface structure are carried on unstable elements (phage, plasmids). In addition, many chromosomal genes affecting surface antigens or appendages involved in pathogenesis are subject to high-frequency variation, enabling the bacterium to adapt rapidly to different ecological niches or to evade host immunological defences. Genetic approaches have greatly increased our appreciation for the sophistication of successful bacterial pathogens, and are rapidly being used to create exciting new vaccines.

Introduction

There are numerous ways in which genetics can be used to analyse bacterial virulence. In this review I shall cover many of these areas briefly, with a few representative examples. These areas include: use of specific mutations to elucidate the importance of particular microbial products in the production of disease; delineation of the roles of extrachromosomal elements (phage and plasmids) in microbial virulence; analysis of the basis of high-frequency variations in cell-surface, and the utility to the bacterial pathogen of such high-frequency variations; and genetic approaches to vaccine development.

Analysis of the roles of specific bacterial virulence factors

Investigators have been interested for a century in the particular attributes of bacterial pathogens that result in illness, usually with the goal of creating vaccines. In some instances, the answer seemed surprisingly simple: the discovery of potent exotoxin such as diphtheria toxin was rapidly followed by the proof that a toxoid vaccine prevented the disease. In many other instances, however, such a straightforward answer has not been forthcoming, and attempts to create vaccines with crude preparations of killed whole cells of many pathogens (e.g. *Staphylococcus aureus*, *Salmonella typhi*, *Vibrio cholera*, *Neisseria gonorrhoeae*) met with modest success at best. Although improved sanitation and public health have markedly reduced the incidence of some bacterial diseases, and antibiotics have markedly reduced the morbidity and mortality consequent to nearly all bacterial diseases, there are still great problems with many of them. Indeed, the increasing prevalence of antibiotic resistance in some bacterial species has increased the need for effective vaccines and therefore for a more thorough knowledge of the molecular pathogenesis of bacterial infections.

The first insights into the pathogenesis of an infectious disease frequently originate in the clinical microbiology laboratory, or from epidemiological considerations. For instance, it is well known that *S. aureus* infects apparently normal hosts, whereas the closely related *S. epidermidis* rarely infects normal hosts. Microbiologists long ago learned that *S. aureus* differs from *S. epidermidis* in a number of properties: the production of coagulase, haemolysins, deoxyribonuclease, and many others. Which of these factors is responsible for the virulence of *S. aureus*? Is any one factor responsible for the virulence, or is virulence the result of the combined effects of multiple factors? In short, which of these factors are causally related to virulence, and which are merely correlated with virulence?

The question can often be answered with reasonable certainty, if two requirements can be met: it must be possible to create experimental conditions where only one bacterial factor is varied at a time, and there must be an appropriate model to test virulence *in vitro*, preferably an animal model that closely reproduces the analogous infection in humans. Modern techniques of genetic manipulation of bacteria, or of production of monoclonal antibodies directed very specifically at a single bacterial antigen, make it relatively easy to accomplish the first goal. Fortunately there are also reasonable animal models of most bacterial infections, although the lack of a really appropriate model infection still impedes progress in some important areas.

Classical methods of genetic analysis including spontaneous or induced mutation, and the standard techniques of gene transfer (transduction, transformation and conjugation) have been used successfully to study virulence factors in many bacteria. For example, Hasegawa & San Clemente (1978) showed that the 50% lethal dose ($l.d._{50}$) of *S. aureus* for mice was increased by over 100-fold by coagulase-negative mutations, compared with the apparently isogenic coagulase-positive parent strain. In contrast, deoxyribonuclease-negative mutations had no effect on mouse virulence. There are, of course, many other potential or actual virulence factors of *S. aureus*; this example merely illustrates an approach to studying the roles of particular factors.

S. aureus also provides an instructive example of the importance of studying the virulence of live bacteria in animal models. Considerable evidence shows that *S. aureus* cell walls are able to activate complement directly, and that the ability of isolated peptidoglycan to activate complement *in vitro* is increased if the peptidoglycan contains attached teichoic acids (Wilkinson *et al.* 1978). Nevertheless, isogenic whole cells that were either normal for teichoic acid or deficient in synthesis of teichoic acid were identical in their ability to activate complement (Wilkinson *et al.* 1981), showing that results *in vitro* with isolated components may give a highly misleading impression of their roles in the pathogenesis of infection. Bacterial cell surfaces are very complicated three-dimensional structures, and their biology is markedly affected by the stearic chemistry and other interactions between several different components.

The use of animal models to study virulence is desirable, but few animal models exactly reproduce human infections. In addition, the use of different animal models may lead to different conclusions. For instance, *Pseudomonas aeruginosa* produces many potentially toxic products, including endotoxin, an exotoxin A that mimics diphtheria toxin in its action, proteases, phospholipases, and frequently a superficial slime layer. Several laboratories have studied the virulence of isogenic mutants of *P. aeruginosa* in animal models. Exotoxin A has been found to be a major virulence factor in all studied model infections (Ohman *et al.* 1980; Woods *et al.* 1982), whereas one of the proteases (elastase) enhances virulence in models of *Pseudomonas* sepsis in burn wounds (Pavlovskis & Wretlind 1979) and in chronic lung infections of rats

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(Woods *et al.* 1982), but has no effect in models of corneal infection (Ohman *et al.* 1980). This last observation indicates that caution should be used in generalizing results obtained in a particular animal model; in this instance, the differing role of elastase in the various model infections was attributed to differences in concentrations of the substrate elastin in different tissues (Ohman *et al.* 1980).

Other studies showed that mutants of *P. aeruginosa* that are unable to produce functional flagella are relatively avirulent in the burned-mouse model (Montie *et al.* 1982), and that alteration of the chemical and antigenic properties of the superficial slime glycolipoprotein by lysogenic conversion results in altered virulence in a mouse model (Dimitracopoulos & Bartell 1979). Thus by the use of relatively simple genetic techniques a beginning has been made in understanding the particular roles of multiple *Pseudomonas* virulence factors; virulence is not determined by a single factor, and the roles played by individual factors in various tissues are undoubtedly different.

Problems with the 'classical' genetic approach

Multiple mutations

The usual objective of a genetic study of the biochemistry and immunobiology of an infectious disease is to create conditions where only a single variable is altered at a time. Unfortunately, many chemically induced mutations result in multiple mutations of the test strain. This problem is particularly severe after mutagenesis with *N*-methyl *N'*-nitro *N*-nitrosoguanidine (NG), but occurs with most chemical mutagens. Even spontaneous mutants cannot safely be assumed to be altered at only a single site, although spontaneous mutants are least likely to bear additional silent mutations in genes other than that under study. Spontaneous mutations are also relatively rare, ordinarily occurring with a frequency of 10^{-6} to 10^{-10}; if there is no method for selection of the desired trait, chemical mutagens may have to be used because frequencies of mutation are many orders of magnitude greater after the use of agents such as NG or ethyl methane sulphonate. As a general rule, mutations that have been induced by the use of chemical mutagens should always be transferred into a non-mutagenized recipient strain before embarking on extensive studies of the biological or biochemical effects of the mutation, so as to remove as many of the unwanted silent mutations of other genes as possible. (This of course supposes that a genetic system exists for the organism under study.)

Linked genes

Classical genetic techniques involve transfer of a relatively small piece of DNA from a donor into a recipient, but linked genes may be transferred simultaneously. Mutations induced by NG are located characteristically in a linked cluster at the site of DNA replication (Cerdá-Olmeda *et al.* 1968), with the result that one can never be absolutely certain that a mutated strain is altered only in the property under study even if it has been 'cleaned up' by the introduction of the mutation into a non-mutagenized recipient.

Related problems may occur when studies are undertaken of naturally occurring mutations in clinical isolates. One example will be illustrative. *Neisseria gonorrhoeae* that cause disseminated (bacteremic) infection are unusually likely to be resistant to the bactericidal effects of pooled normal human serum (Schoolnik *et al.* 1976), and to be of a particular outer membrane protein I immunotype (Hildebrandt & Buchanan 1978). It therefore was of considerable interest when genetic loci affecting protein I and serum resistance were said to be identical, because the two

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traits (protein I and serum resistance) were apparently inseparable when a small number of transformants were analysed (Hildebrandt *et al.* 1978). Subsequent genetic studies of several other gonococcal strains, however, showed that loci affecting the structure and immunotype of protein I (designated *nmp* for new membrane protein) and serum resistance (designated *sac* for serum antibody and complement) were merely closely linked; they could be separated if a larger number of transformants were analysed (Cannon *et al.* 1981). Studies of clinical isolates have so far defined a closely linked cluster of at least two *sac* loci that determine different degrees of serum resistance (Shafer *et al.* 1982). The *sac* loci do not affect the structure of protein I as determined by electrophoresis on sodium dodecyl sulphate polyacrylamide gels, and do not affect the gross structure of lipopolysaccharide (Cannon *et al.* 1981; Shafer *et al.* 1982). The mechanisms of serum resistance are uncertain. The relevant point in this discussion is that performance of a little genetics may be hazardous; one must be as rigorous as possible before deciding that two phenotypic traits are due to mutations of a single gene. (There are examples of pleiotropic mutations that have multiple phenotypic effects (Guymon *et al.* 1978).)

Other approaches

Molecular cloning and transposon mutagenesis

The advent of recombinant DNA methods has provided another and in some respects better means for studying bacterial virulence. The literature is already replete with examples of the use of molecular cloning to isolate and manipulate bacterial virulence factors (see Falkow, this symposium). The principal advantage of this method is its precision. It is possible to isolate a defined (and very small) piece of DNA, to determine its sequence rapidly, and to create specific alterations at exact sites within the gene. The availability of a cloned gene allows rapid and relatively unambiguous determination of the structure of a protein virulence factor, and also makes it possible to search for related genes in clinical isolates by DNA–DNA hybridization. An excellent manual describing most of the relevant techniques is available (Maniatis *et al.* 1982).

Molecular cloning is not, however, a panacea. It certainly is easier to clone genes for proteins than for other substances such as capsules and lipopolysaccharides. There may be a variety of problems in stable expression of reasonable quantities of the cloned gene product. A specific probe must be available to identify the cloned gene or its product. These problems, and some applications of cloning and of transposon-mutagenesis to studies of bacterial virulence are discussed in the paper by Falkow (this symposium).

Monoclonal antibodies

Another approach is provided by the use of monospecific or monoclonal antibodies. There has been relatively little use so far of monoclonal antibodies in studies of bacterial virulence, but this is bound to change as more monoclonal antibodies become available. Monoclonal antibodies recognize highly specific epitopes, and cause few problems of cross-reactions. The importance of monoclonal antibodies for the geneticist is their utility in obtaining mutants of structural elements whose function is not known, or which are not readily measured or assayed. They are useful for *scoring* for the loss or acquisition of a particular antigen, because large numbers of colonies can be tested efficiently by adaptations of the colony-blot immunoassay developed by Henning *et al.* (1979). Monoclonal antibodies against integral outer-membrane proteins are often bactericidal, at least in *N. gonorrhoeae* (J. Cannon, personal communication),

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and can therefore be used directly to *select* variants lacking the target antigen for the bactericidal monoclonal antibody. Related techniques have been used recently to select a large number of very interesting mutants of poliovirus (Minor *et al.* 1983).

Monoclonal antibodies can also be infused into animals to help determine whether the target antigen is involved in virulence. Hunter *et al.* (1982) used specific anticapsular human monoclonal antibodies to protect against lethal infection by *Haemophilus influenzae* in an infant rat model, confirming the specific role of capsule in virulence, which had been strongly implicated previously by a number of criteria including studies in animal models of transformants with different capsular antigens (Moxon & Vaughn 1981; Roberts *et al.* 1981). Even more interesting is the study by Hansen *et al.* (1982), who showed that a 39 kDa outer membrane protein of encapsulated type B *Haemophilus influenzae* apparently is involved in virulence for infant rats, because an anti-39 kDa protein monoclonal antibody resulted in immunospecific protection. In general, although much is known about the basis for increased virulence of encapsulated bacteria, relatively little is known about the roles of most outer membrane proteins of Gram-negative bacteria in virulence.

TABLE 1. VIRULENCE-ASSOCIATED GENES CARRIED ON NON-CHROMOSOMAL REPLICONS (PARTIAL LIST)

	reference
bacteriophage	
Clostridium diphtheriae toxin	Groman & Eaton (1955)
Clostridium botulinum toxins	Hariharan & Mitchell (1976)
Vibrio cholerae serotype	Ogg *et al.* (1978)
Escherichia coli heat labile enterotoxin	Takeda & Murphy (1978)
Pseudomonas aeruginosa glycolipid slime	Dimitracopoulos & Bartell (1979)
Klebsiella pneumoniae adherence ligand	Pruzzo *et al.* (1980)
Streptococcus pyogenes pyrogenic exotoxin	Zabriskie (1964)
plasmids	
Escherichia coli	
heat-labile, heat-stable enterotoxins	see review by Elwell & Shipley (1980)
adherence ligands	
haemolysin	Smith & Halls (1967)
serum resistance	Taylor & Robinson (1980)
iron uptake	Williams & Warner (1980)
Shigella sonnei O antigen	Sansonetti *et al.* (1981)
Shigella flexneri epithelial cell penetration	Sansonetti *et al.* (1982)
Salmonella typhimurium adhesins, invasion	Jones *et al.* (1982)
Yersinia sp. virulence, outer membrane proteins	Zink *et al.* (1980)
Staphylococcus aureus exfoliative toxin	Rogolsky *et al.* (1976)
Clostridium tetani toxin	Laird *et al.* (1980)

GENES FOR BACTERIAL VIRULENCE ARE OFTEN NON-CHROMOSOMAL

There is a long and still growing list of the number of known genes that affect bacterial virulence and that are carried on extrachromosomal replicons, either bacteriophage or plasmids (table 1). Plasmids are known that carry genes for a number of different virulence factors, sometimes in association with antibiotic resistance genes (see Elwell & Shipley 1980). Undoubtedly the great majority of genes affecting virulence are chromosomal, but the number of known non-chromosomal virulence genes is impressive, and suggests that there may be a selective advantage to carrying virulence genes on a phage or plasmid. One obvious advantage is the potential for increased spread of the non-chromosomal virulence genes to other bacteria of the same or related species. This may be particularly important for plasmid-borne genes,

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because some plasmids have a broad host range and can replicate relatively stably in many different bacterial species. Another potential advantage of extrachromosomal virulence genes is an increased number of copies of the virulence gene per cell, particularly for genes carried on plasmids that replicate under relaxed control. Consistent with this is the recent suggestion (not yet rigorously proven) that *Salmonella typhimurium* is more adhesive and mouse-virulent when a 60 MDa plasmid is present as a freely replicating, autonomous cytoplasmic element than when the same plasmid is integrated into the chromosome (Jones *et al.* 1982). The relative abundance of virulence genes on extrachromosomal elements may also be due to the fact that non-chromosomal DNA represents a site where random mutations may occur without deleterious consequences to the cell. When a rare mutation occurs that increases the survival capacity of the organism, it will be selected.

Genetics of cell-surface variation

There is another rapidly growing and very interesting field of study of the genetics of pathogens: the basis for high-frequency variation in crucial cell-surface antigens. It has been appreciated for some time that certain bacteria and protozoans are capable of rapidly altering their cell surface, at rates too high to be explained by ordinary mutations. Many of these variations are bidirectional, and thus cannot be explained by high-frequency loss of a phage or plasmid. Examples of such variations are provided by *Salmonella* flagellar antigens (Zieg *et al.* 1977), trypanosomal surface antigens (Hoeijmakers *et al.* 1980), *E. coli* type 1 pili (fimbriae) (Eisenstein 1981), and *N. gonorrhoeae* type 1 pili (Meyer *et al.* 1982) and outer membrane protein II (Mayer 1982). In some instances the genetic basis for the variation is well known. Inversion of a short (995 base pair) chromosomal DNA sequence results in the expression of one of two types of *Salmonella* flagella, depending on the direction of insertion of this invertable element (Zieg *et al.* 1977). Variation in expression of *E. coli* type 1 pili is under transcriptional control and is very rapid, alternating between Pil^+ (pilus-expressing) and Pil^- (non-pilus-expressing) at a nearly bidirectionally equal rate of about 10^{-3} (Eisenstein 1981), perhaps owing to a mechanism similar to *Salmonella* flagellar variations.

Variations of gonococcal pili are complex because they undergo both an 'on–off' variation ($Pil^+ \rightleftarrows Pil^-$) as well as a variation between multiple structurally and antigenically distinct forms of pili (Pil α, Pil β, Pil γ, etc.) (Lambden *et al.* 1981). Although the genetic basis for gonococcal pilus variation is not well understood, it involves a rearrangement of chromosomal DNA (Meyer *et al.* 1982). Another complex variation is the alteration of gonococcal outer membrane proteins designed P.II, which belong to a family of six or more closely related host-modifiable proteins associated with differences in pathogenicity (Swanson 1978; James & Swanson 1978). These proteins vary between 'on–off' ($P.II^+$ and $P.II^-$), and between different forms of P.II (P.IIa, P.IIb, etc.) (Lambden *et al.* 1979). A single cell may express only one or two of the P.II proteins at one time (J. Cannon, personal communication). The switch from $P.II^+$ to $P.II^-$ or from $P.II^-$ to $P.II^+$ occurs with a frequency of about 10^{-3} (Mayer 1982). At present, almost nothing is known about the genetic basis for P.II variation, except that the P.II structural genes are chromosomal (J. Cannon, personal communication).

Why should bacteria have evolved mechanisms for such high-frequency variation of chromosomal genes controlling cell-surface antigens? There is one rather obvious explanation: it provides the bacteria with a means for relatively rapid escape from antigen-specific host

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defences (Lambden *et al.* 1982). A second less intuitively obvious explanation is also emerging, based on the concept that certain cell-surface structures are advantageous at certain stages of infection (type 1 pili mediate mucosal attachment (Swanson 1973)), but may be disadvantageous at other stages (type 1 Salmonella pili increase rates of clearance from the blood by the liver (Leunk & Moon 1982)). For the bacterium, it would be advantageous to be able to shift rapidly between alternative states (e.g. Pil^+ and Pil^-) so that there are representatives of each phenotype in even a relatively small inoculum, thereby providing variants particularly well adapted to different niches within the host. For the investigator, the phenomenon of high-frequency cell-surface variation means that it is usually virtually impossible to grow a large population of identical cells in the laboratory, even under carefully controlled conditions such as growth in a chemostat. The implications of such variations are great, and it seems highly likely that other examples of related variations will be found in many other bacterial pathogens in the future.

Vaccine development

Microbial geneticists are contributing to yet another important aspect of bacterial pathogenesis, the development of new and more refined vaccines. Several different strategies are being used. In one approach, mutations are introduced that result in a 'conditional lethal' phenotype, with the result that the organism only grows in a limited fashion in the host. One highly noteworthy example is the now famous galactose epimerase (*galE*) mutation of *Salmonella typhi*. This mutation results in an inability to grow in the absence of galactose; provision of galactose results in temporary growth followed by cell death due to the accumulation of toxic products of galactose metabolism. The net result is sufficient growth *in vivo* to stimulate protective immunity, but not enough growth to cause disease. The *galE* mutant *S. typhi* has been shown to be an effective live vaccine in volunteers (Gilman *et al.* 1977) and in an extensive field trial (Wahdan *et al.* 1980), a marvellous practical outgrowth of fundamental studies of microbial biochemical genetics. Recent studies have shown that a *Shigella* virulence plasmid can be introduced into the *galE S. typhi* strain, with expression of the *Shigella* virulence antigens (Formal *et al.* 1981). Administration of a live *galE S. typhi–Shigella* hybrid to animals resulted in specific immunity to both *S. typi* and *Shigella* (Formal *et al.* 1981).

Other new vaccines are being created by the construction of non-reverting mutations of specific virulence factors, e.g. enterotoxins (Dallas *et al.* 1979). Techniques of molecular cloning are particularly useful, in that specific deletions can be constructed relatively easily once a gene for a virulence factor is cloned. Unlike spontaneous or induced point mutations, deletions do not revert; immunogenic but avirulent deletion mutants should be very safe live vaccines.

Concluding remarks

Techniques of bacterial genetics have broad applicability to the field of bacterial pathogenesis. This short review only illustrates some of these uses. Other important areas such as the genetics of the susceptibility of the host to particular pathogens (Skamene *et al.* 1982) have been omitted entirely. It is remarkable how many investigators have used genetics recently to study bacterial pathogenesis. Genetic approaches are certainly not the only fruitful ones, and they are not without limitations and problems, but they do provide very powerful tools to dissect the infectious process.

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