Annu. Rev. Microbiol. 1999. 53:129–54
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IN VIVO GENETIC ANALYSIS OF BACTERIAL VIRULENCE

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Key Words in vivo expression technology, signature-tagged mutagenesis, differential fluorescence induction, pathogenesis, GAMBIT

■ **Abstract** In vitro assays contribute greatly to our understanding of bacterial pathogenesis, but they frequently cannot replicate the complex environment encountered by pathogens during infection. The information gained from such studies is therefore limited. In vivo models, on the other hand, can be difficult to use, and this has to some extent diminished the incentive to perform studies in living animals. However, several recently developed techniques permit in vivo examination of many genes simultaneously. Most of these methods fall into two broad classes: in vivo expression technology and signature-tagged mutagenesis. In vivo expression technology is a promoter-trap strategy designed to identify genes whose expression is induced in a specific environment, typically that encountered in a host. Signature-tagged mutagenesis uses comparative hybridization to isolate mutants unable to survive specified environmental conditions and has been used to identify genes critical for survival in the host. Both approaches have so far been used exclusively for investigating pathogen-host interactions, but they should be easily adaptable to the study of other processes.

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0066-4227/99/1001-0129$08.00

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INTRODUCTION

The virulence of bacterial pathogens (their ability to produce morbidity and mortality in a host) is a complex, multifactorial process requiring the coordinated activity of many bacterial gene products. Infections may be described generally as proceeding in a sequence that begins with attachment to and colonization of the host, followed in the case of some pathogens by invasion of host tissues or cells. To multiply and persist within the host, a pathogen must then be able to circumvent the host's immune system and obtain nutrients for itself. Exit from the host and transmission to new hosts are subsequent stages in the infectious cycle, and a pathogen may at any point during infection produce factors that cause damage to the host (25, 38).

A variety of in vitro systems have been developed that simulate certain aspects of the infectious process, enabling the development of screens to study bacterial gene expression and the behavior of mutant strains in physiological conditions that reflect the situation in vivo (24). These include the use of specific culture conditions to mimic the host environment and tissue culture assays for adhesion, invasion, or cytotoxicity. For example, studies of bacterial responses to changes in pH (55), temperature (44, 49), and iron levels (10, 29, 39), and analysis of host cell invasion (27, 51) and survival in macrophages (7, 12, 13, 23, 26) have all been used to identify and characterize bacterial virulence determinants.

In vitro assays have been enormously useful and continue to provide much information on the mechanisms of bacterial pathogenesis, but it is obvious that they cannot accurately reproduce all aspects of the host-pathogen interaction. A pathogen may encounter several radically different environments in the host, and it may therefore have very different requirements at various points during infection, particularly in the context of a developing immune response. Consequently, a gene that seems important in in vitro studies may not be important in vivo, and genes that appear unimportant in an in vitro assay may play a critical role during a natural infection.

For these reasons, in vivo experimental models are highly desirable. They permit direct assessment of a pathogen's ability to colonize and survive in a living host and to cause disease or damage. Animal models nevertheless have their own limitations, being generally labor intensive, expensive, and otherwise unwieldy, and these issues present a considerable barrier to undertaking large-scale in vivo experiments. This is perhaps one reason that many searches for novel virulence

determinants have focused on identifying factors that are coregulated with known virulence determinants, rather than attempting to conduct generalized screens in animals. Nevertheless, some screening of individual mutant strains for altered virulence has been carried out on a limited scale with animal infection models, using either randomly chosen transposon mutants (11) or strains affected in cell surface or extracellular proteins (48).

Recently, however, several methods have been developed that greatly simplify in vivo analysis of large numbers of strains. A number of these can be classified as in vivo expression technology (IVET) methods. These are promoter-trap strategies designed to identify promoters that are specifically activated in the host, and many IVET procedures permit positive selection for such promoters. Another method that has been used to perform in vivo analysis is signature-tagged mutagenesis (STM), which relies on comparative hybridization to identify mutants unable to survive in the host. In this review, we summarize adaptations of these techniques that have been used in different bacteria, compare the genes identified by IVET and STM in *Salmonella typhimurium*, *Staphylococcus aureus*, and *Vibrio cholerae*, and discuss the advantages and disadvantages of the two approaches. Possible future development and applications of these and several recently developed methods are also considered.

EARLY SCREENS FOR IN VIVO-INDUCED GENES

Upon entering the host, many pathogenic organisms find themselves in a situation that must differ significantly from any encountered in the environmental reservoir. Bacteria respond to this change in circumstances by modulating their patterns of gene expression accordingly, downregulating the expression of genes that are no longer necessary, and upregulating those that are specifically required for survival in the host (e.g. nutrient acquisition or evasion of host defenses). It therefore seemed probable that at least some in vivo-induced genes would play a critical role in pathogenesis, and several promoter-trap strategies were used to identify genetic loci whose expression is induced in host environments.

Conceptually, finding in vivo-induced genes is potentially a simple process if it is possible to generate appropriately selectable or screenable gene fusions and obtain a host organism that is amenable to brute-force screening. For example, one of the first screens for host-induced genes was carried out in the plant pathogen *Xanthomonas campestris* (56). A library of *X. campestris* DNA fusions to a promoterless chloramphenicol resistance gene was generated and introduced into *X. campestris* on plasmids. Eleven hundred of the resultant strains were individually tested for ability to produce disease symptoms in chloramphenicol-treated turnip seedlings. Of the 19 strains found to be virulent in treated seedlings, 14 were also highly sensitive to chloramphenicol in vitro, indicating that these 14 strains harbored plasmids carrying host-inducible fusions. Mutations were subsequently created in and around two of the host-inducible genes, and the mutant strains were

tested for effects on pathogenicity. Mutations in one of the genes led to delayed symptom expression in plants (57), whereas mutations in the other gene had no discernible phenotype (58).

Another example of brute-force screens for in vivo-induced genes involved individual scoring of 2550 *Listeria monocytogenes* Tn*917-lac* mutants for genes expressed at higher levels in macrophage-like cells than in laboratory medium (37). This resulted in the identification of five genes that had as much as 100-fold induction within macrophages. Three of these genes were nucleotide biosynthetic genes, one (*arpJ*) was involved in arginine uptake, and the fifth was *plcA*, the gene encoding the previously identified virulence factor phosphatidylinositol-phospholipase C (16). Mutations in the nucleotide biosynthetic genes did not result in increased LD_{50} values in mice, although the purine mutation tested did reduce the number of bacteria recoverable from the liver. The *arpJ* mutation caused a twofold increase in LD_{50} as well as a decrease in bacterial load in the liver, whereas the *plcA* mutant showed a 25-fold increase in LD_{50} (46) and decreased bacterial load in both liver and spleen.

These results provide excellent examples of how promoter-trap screens can identify in vivo-induced genes in host-pathogen models amenable to selection or biochemical assay. However, since each strain carrying a given gene fusion was tested individually, the procedures described above were relatively laborious. This was especially true in the *Xanthomonas* study, in which each strain was inoculated by hand into antibiotic-treated seedlings. Therefore, although the goal of identifying host-inducible genes was achieved in both cases, it was clear that significant reductions in labor would represent a critical advance in the development of similar methods for studying bacterial gene expression in the host.

IN VIVO EXPRESSION TECHNOLOGY

In the last five years, a variety of additional methods have been formulated to isolate genes whose expression is induced in the host, all of which have increased efficiency compared with the examples discussed above. Such techniques were generally termed "in vivo expression technology" (IVET) methods, and although initial interest was understandably focused on host-induced genes, IVET could presumably be adapted to study the induction of microbial genes in response to any condition.

The first IVET methods used promoterless reporter genes whose products confer a phenotype that can be positively selected in the host (42). Both auxotrophic and antibiotic selections were used to this end. The recently developed differential fluorescence induction (DFI) method (67) also permits positive selection for host-induced promoters, although the selection is carried out by fluorescence-activated cell sorting (FACS) of organisms recovered from host cells or animals. Resolvase IVET uses genetic recombination as a reporter activity and requires screening for host-induced promoters after bacteria are recovered from host tissues. Its

advantage is that it can in theory detect promoters that are only weakly or transiently induced during infection (14, 15).

Auxotrophic Selection

The original IVET selection (42) was performed by creating transcriptional fusions of random fragments of the *S. typhimurium* chromosome with a promoterless *purA* gene and introducing this library onto the chromosome of an *S. typhimurium* Δ*purA* strain via homologous recombination at the chromosomal fragment (Figure 1). Because purines are limiting for growth of *S. typhimurium* in the mouse, only those strains expressing *purA* from fused promoters would survive. It should be noted that the integration event resulting from a single crossover does not lead to disruption of the wild-type locus on the chromosome, thereby permitting analysis of genes essential for growth in vivo. Bacteria representing the pool of chromosomal fusions were then injected intraperitoneally into BALB/c mice, and the surviving pools were recovered 3 days later and screened on laboratory medium for clones with low promoter activity. Several strains carrying promoters meeting the criteria of in vivo expression and in vitro inactivity were, on subsequent analysis, found to have severe virulence defects as assayed by oral LD_{50}, thereby validating the ability of IVET to isolate virulence genes.

Similar IVET selections were carried out in *Pseudomonas aeruginosa*, both in vivo in BALB/c mice (70) and in vitro to find genes induced by respiratory mucus collected from cystic fibrosis (CF) patients (69). The latter study was undertaken on the premise that *P. aeruginosa* isolates from CF patients are phenotypically different from isolates from natural environments and that CF respiratory mucus might contain substances that induce expression of CF patient-specific virulence factors. Both selections successfully identified novel loci specifically induced under their respective conditions, including genes with no known homologs. Two specific loci were identified independently by both studies. One of these genes encoded the proposed virulence determinant FptA, a protein involved in iron acquisition (5), and the gene product of the other, *np20*, was similar to ferric uptake regulatory proteins. Insertional disruption of *np20* in a wild-type genetic background caused an ~100-fold increase in LD_{50}. The other mouse-induced *P. aeruginosa* loci displaying homology to known proteins appeared to fall into the two general categories of gene regulation and amino acid biosynthesis. Further information regarding the virulence contributions of these genes and the contributions of the mouse *ivi* genes without known homologs is not yet available.

Antibiotic Selection

The IVET method described above obviously requires the existence of an attenuating and complementable auxotrophy, which unfortunately may not be readily available in all microbial systems. However, a variation on the basic principle was established in which expression of the reporter gene provided resistance to the antibiotic chloramphenicol, which could be administered to the host. Using this

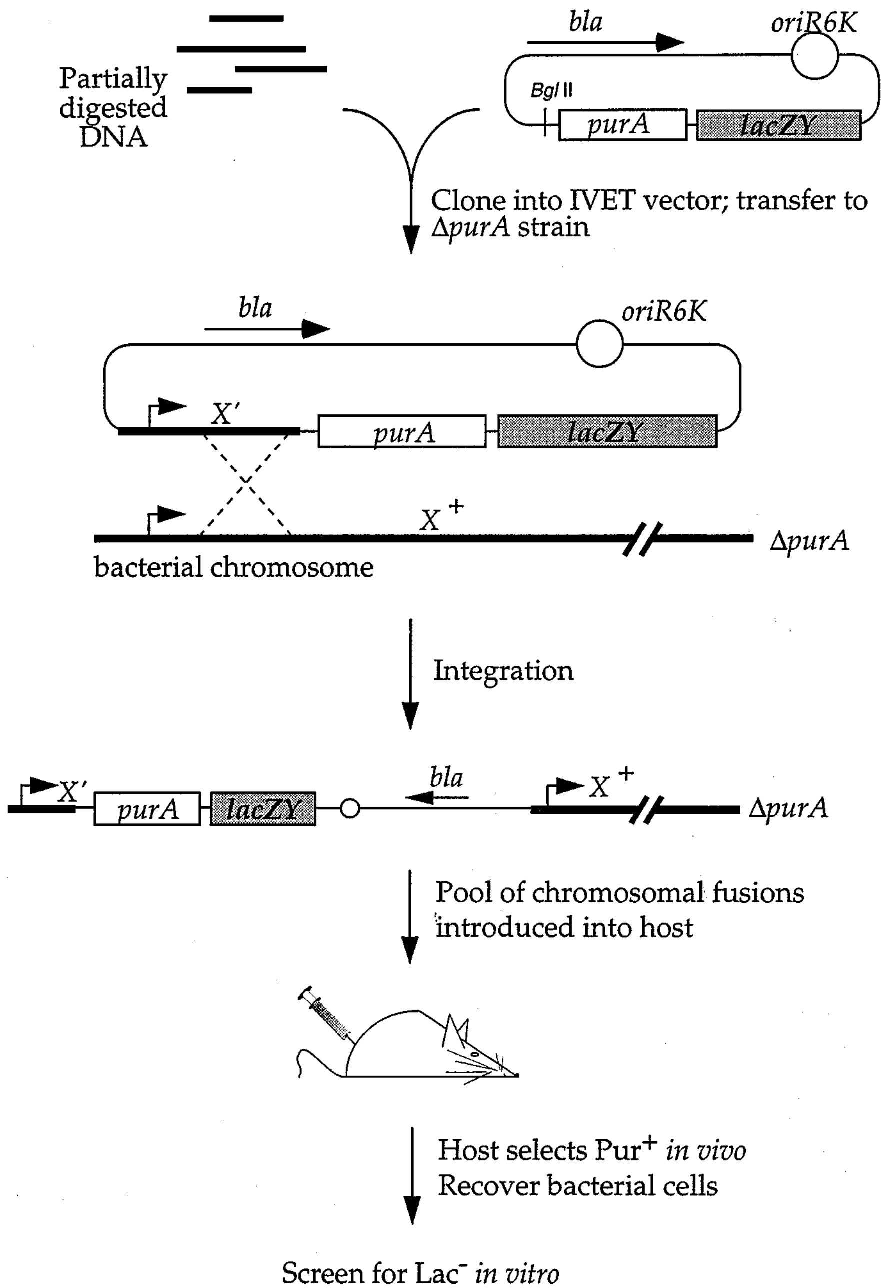


Figure 1 Schematic representation of auxotrophic IVET selection strategy. Adapted from Reference 42.

method, it should be possible to carry out selection for *ivi* genes in any tissue in which the antibiotic concentration can be made sufficiently high to select against strains not expressing the resistance gene. Adjustment of the antibiotic dosage may permit isolation of *ivi* promoters with different levels of activity, and variation of the timing of antibiotic administration might allow investigators to identify *ivi* genes that are expressed at a particular time or place during infection.

The first application of antibiotic-based *ivi* selection was also carried out in *S. typhimurium*, in both BALB/c mice and in cultured macrophages (43). Taken together, the *purA* (42) and antibiotic IVET selections identified >100 *ivi* genes in *S. typhimurium*. Several of these were known virulence determinants, but more than half had either no homologs or none with known function. For example, one of the in vivo-induced genes identified by Heithoff et al (31) was *phoP*, which is known to autoregulate its own expression as well as the expression of multiple virulence genes that are induced after invasion into macrophages (31, 50). Mutations in many of the *ivi* genes had no significant effect on LD_{50}, but some mutant strains showed reduced ability to persist in the spleen (31).

Thirty of the *S. typhimurium ivi* genes identified to date are located in regions of atypical base composition. Hybridization analysis showed that these *ivi*-containing regions are specific to the *Salmonellae* but that several are serovar specific. Although some were present in all salmonellae, others were present only in broad host-range serovars (*S. typhimurium* and *S. newport*), and a few were found in all serovars except the host-adapted serovar *S. typhi*. Two of the regions also contain mobile genetic elements or insertionlike sequences, and deletion of certain regions resulted in colonization defects as assessed by competition assays in BALB/c mice. These observations raise the possibility that these regions might have been acquired by horizontal transmission and may have contributed to the evolution of serovars with different host and tissue specificities (21).

Antibiotic-based IVET selection was also successfully used to detect *ivi* genes or host-responsive elements (*hre*) in *Yersinia enterocolitica* (71). Selection was performed in the Peyer's patches of chloramphenicol-treated mice after peroral infection, and the subset of prototrophic strains that were unable to grow on laboratory medium containing chloramphenicol was retained for further analysis. The fusions in these 404 strains were defined as *hre* fusions and were found to fall into 61 different allelic groups. Sequence analysis of 48 *hre* genes showed that about half had significant similarity to known genes, a few were similar to genes with unknown function, and 18 had no similarity to any sequence in public DNA and protein databases. Insertion mutations were constructed in four *hre* genes, and these mutants demonstrated increased LD_{50}, decreased persistence in host tissues, or both.

Genetic Recombination as a Reporter for In Vivo Activity

The paramount advantage of the preceding IVET variations (auxotrophic complementation or antibiotic selection) is the use of positive selection to isolate *ivi* gene fusions from a pool of fusion strains, thereby largely circumventing the labor-

intensive nature of individually screening for such loci. However, both methods favor the identification of genes that are expressed at high levels throughout the infection, because the stringent selective pressures would tend to prevent the survival of strains with fusions to promoters that are expressed weakly or only transiently during infection. Stringent selections might also favor the isolation of promoters that had mutated to higher activity during infection.

To address this problem, an IVET system was developed in which the reporter is $\gamma\delta$ resolvase, which catalyzes irreversible recombination between specific DNA sequences, termed *res* sites. By constructing a system in which resolvase activity results in permanent excision from the chromosome of a tetracycline resistance gene flanked by *res* sites (Figure 2), this method permits detection of promoter activity even if the promoter is active only briefly during infection. Any expression of the resolvase reporter results in a heritable change (i.e. conversion from tetracycline resistance to tetracycline sensitivity) that can be detected by replica plate screening after the bacteria are recovered from the animal (14). Although the resolvase IVET method does not have the benefits of positive selection, theoretically it should be much more sensitive than the previous IVET systems. On the other hand, it may not be able to distinguish between strong and weak induction.

The application of resolvase IVET in *V. cholerae* led to the identification of 13 *ivi* fusions (15). Analysis of the sequences fused to the resolvase reporter determined that some were homologous to genes known to be involved in amino acid biosynthesis and general metabolism, whereas others either had homologs with unknown function or no homologs at all. Two *ivi* fusions appeared to be to antisense transcripts whose gene products are involved in cell motility. Insertion mutants of all 13 loci were tested in infant mouse competition assays, and three *ivi* mutants demonstrated moderate but reproducible colonization defects.

Resolvase IVET was also used in the gram-positive bacterium *Staphylococcus aureus* (40). Owing to the lack of a suitable stable integrating plasmid, the fusion library was not recombined onto the chromosome. A total of 45 *ivi* genes were identified by using the murine renal abscess model. Several were previously known staphylococcal genes, including *agrA*, which is involved in regulation of several virulence factors and is known to be autoregulated (35, 53). The remaining *ivi* genes either had similarity to nonstaphylococcal genes or had no known similarities. Eleven *ivi* genes, representing all three classes, were mutated in the parental genetic background and tested for virulence, and seven of these mutants showed reduced ability to persist in the mouse. Six of these seven attenuating mutations were in genes without homologs in public databases.

Differential Fluorescence Induction A promising new method for identifying genes induced during infection is DFI (66, 67). Developed in the *S. typhimurium* system, DFI uses expression of green fluorescent protein as the reporter for promoter activity and relies on FACS to carry out the selection for active gene fusions. Random fragments of chromosomal *S. typhimurium* DNA were cloned upstream

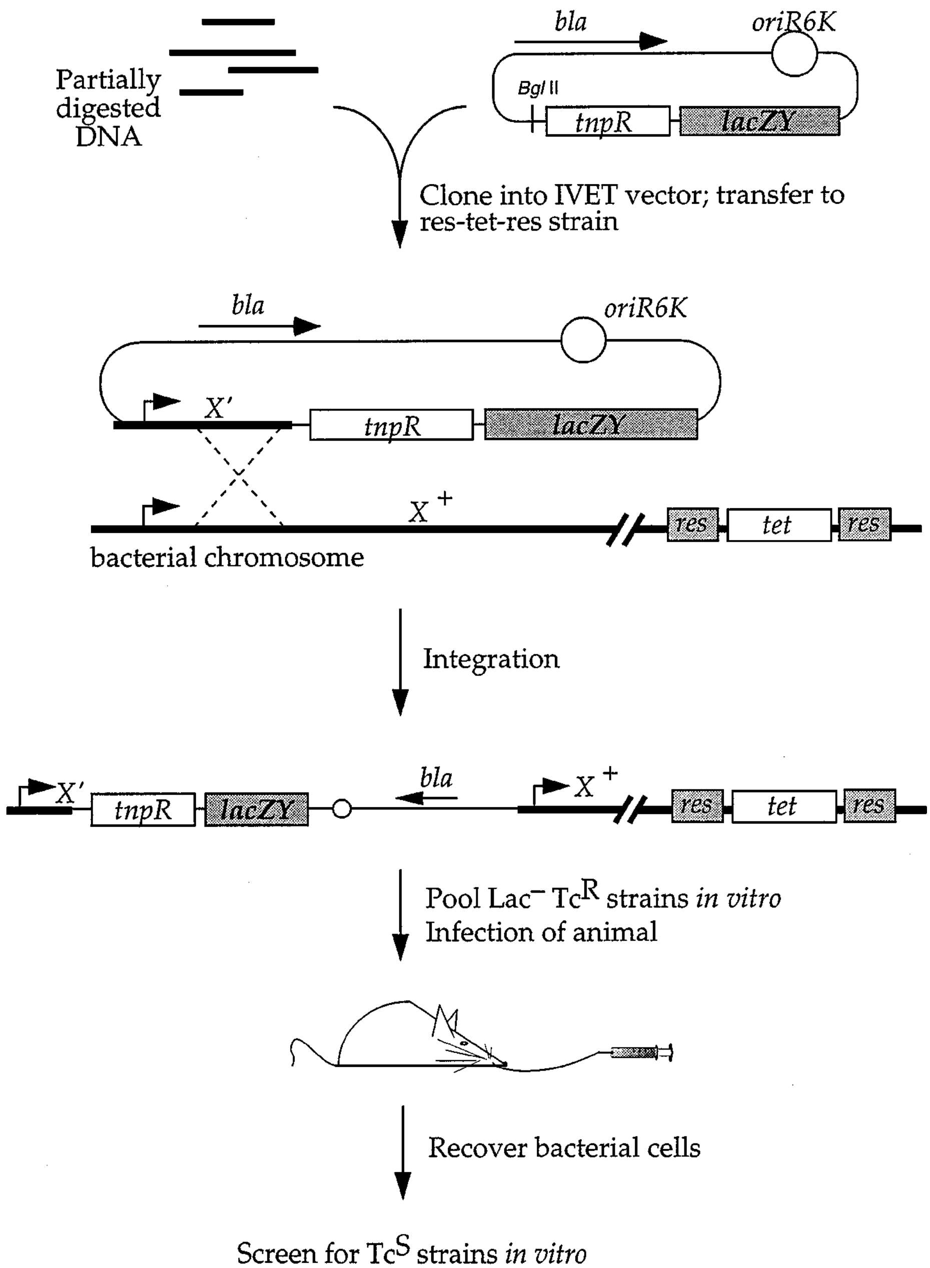


Figure 2 Schematic representation of resolvase IVET strategy. Adapted from Reference 15.

of a promoterless *gfp* gene, and the resultant library was introduced into *S. typhimurium*. To reduce technical difficulties, the library of fusions was maintained on plasmids and not recombined onto the chromosome. After the library of clones was used to infect macrophages, FACS was used to isolate macrophages containing bacteria with active *gfp* fusions. These bacteria were recovered from the macrophages, grown in tissue culture medium, and then re-sorted to obtain clones with low fluorescence (Figure 3). As many as 50% of the promoters thus isolated were confirmed to have host cell-dependent activity on subsequent analysis.

Of 14 macrophage-inducible genes identified by DFI, 8 had bacterial homologs of known function, some of which had previously described roles in virulence. The remaining six genes either had no known bacterial homologs or had homologs with no known function. At least two of these novel loci contribute to virulence, as determined in competition assays testing spleen colonization in BALB/c mice, and both of these loci were regulated by the PhoP/PhoQ two-component regulatory system, which modulates the expression of several macrophage-inducible virulence factors in *S. typhimurium* (4, 8, 30, 50).

SIGNATURE-TAGGED MUTAGENESIS

A different approach to studying bacterial pathogenesis in vivo is STM, a comparative hybridization technique that uses a collection of transposons, each one modified by the incorporation of a different DNA sequence tag. The tags are short DNA segments that contain a 40-bp variable central region flanked by invariant "arms" that facilitate the coamplification and labeling of the central portions by polymerase chain reaction (PCR). When the tagged transposons are used to mutagenize an organism, each individual mutant can in theory be distinguished from every other mutant based on the different tags carried by the transposons in its genome. The use of DNA tags to monitor the fate of different cells in a mixed population was originally used to study the distribution of neuronal clones in the cerebral cortex, by employing retroviruses marked with DNA segments of different sizes and restriction patterns (68).

In STM, mutagenized bacterial strains are stored individually in arrays (usually in the wells of microtiter dishes), and colony or dot blots are made from these arrays. Pools of mutants are then subjected to a selective process such as infection of an animal, and PCR is used to prepare labeled probes representing the tags present in the preselection (input) and postselection (output) pools. Hybridization of the tags from the input and output pools to the colony or dot blots permits the identification of mutants that are unable to survive the selective process, because the tags carried by these mutants will not be present in the output pools. These strains can then be recovered from the original arrays (Figure 4), and the nucleotide sequence of DNA flanking the transposon insertion point can be determined.

In the original method, the suitability of tags was checked before use by amplification, labeling, and hybridization to colony blots representing the tags used

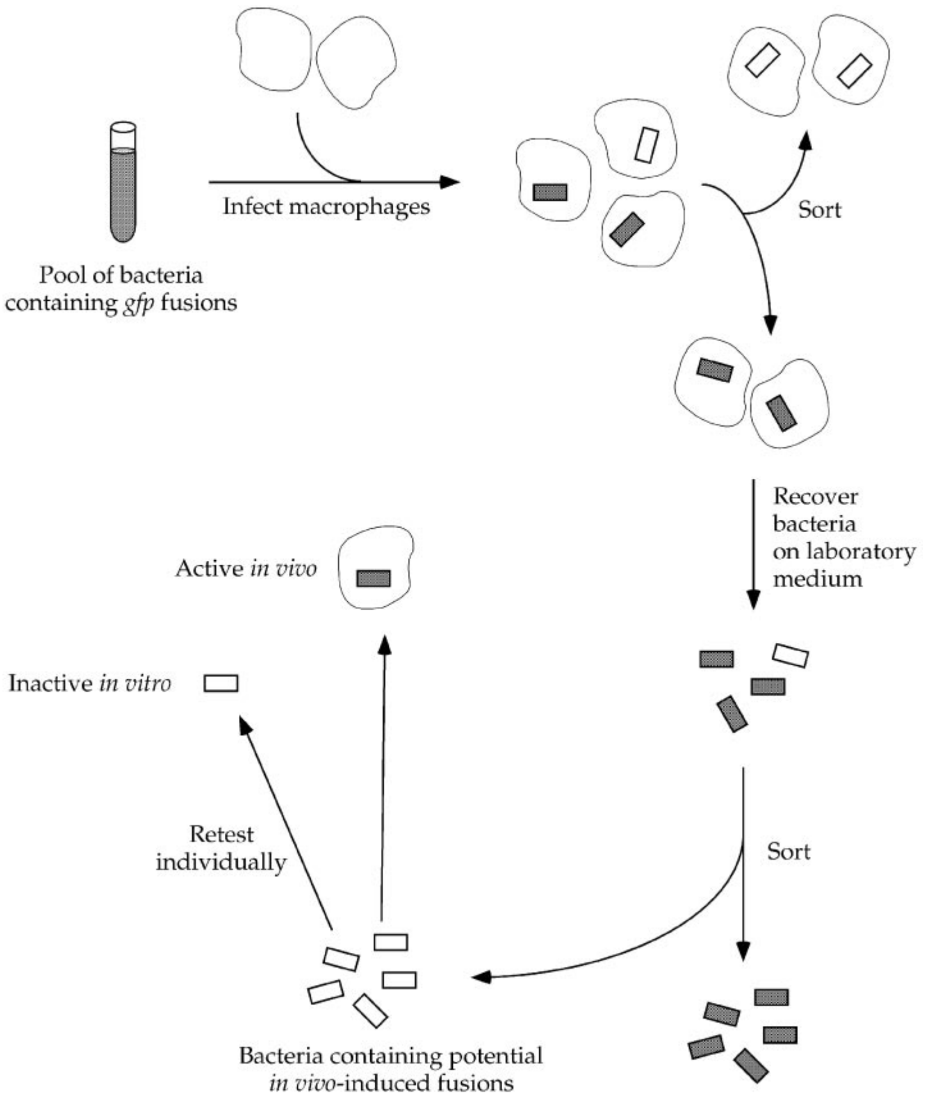


Figure 3 Schematic representation of DFI strategy, as used for isolation of macrophage-induced genes. Adapted from material kindly supplied by T McDaniels and S Falkow (Stanford University).

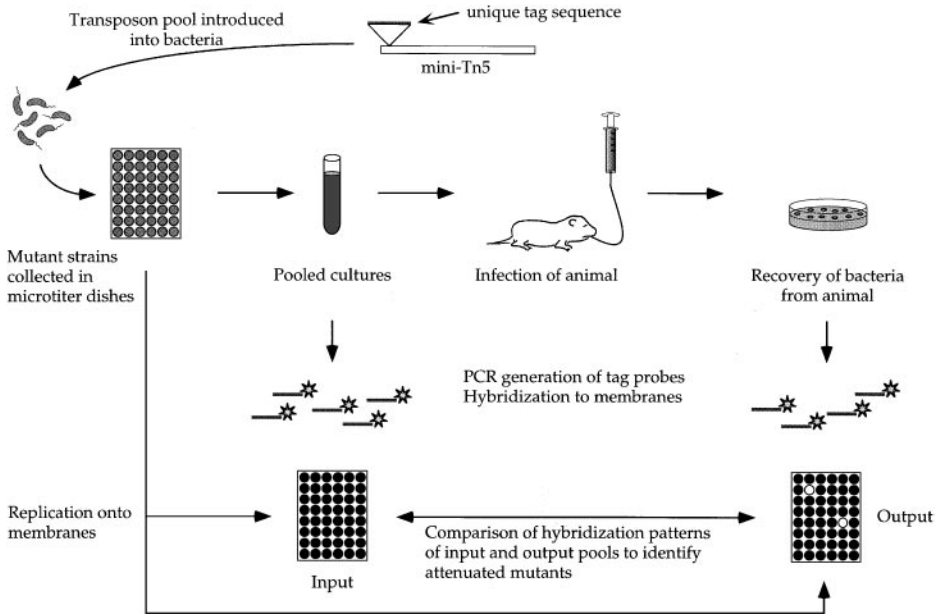


Figure 4 Schematic representation of the original STM strategy.

to make the probes. Mutants whose tags failed to yield clear signals on autoradiograms were discarded, and those that gave good signals were reassembled into new pools for animal infection studies (32). The method was subsequently modified to avoid this prescreening process (45). In this version of STM, a series of tagged transposons is selected before mutagenesis, based on efficient tag amplification and labeling and lack of cross-hybridization to other tags. These modified transposons are then used separately to generate a large number of bacterial mutants that are arrayed based on the tags they carry (Figure 5). Because the same tags can be used to generate an infinite number of mutants, the need to prescreen mutant strains for the suitability of the tags they carry is obviated. A second advantage is that, because the identity of the tag in each mutant is known, hybridization analysis can be done by plasmid or tag DNA dot blots rather than colony blots. This increases the sensitivity of the assay and allows the use of nonradioactive detection methods (45).

STM relies on the ability of the pathogen in question to replicate in vivo as a mixed population and can be expected to identify only virulence genes whose mutant phenotypes cannot be trans-complemented by other virulent strains present in the same inoculum. When STM is applied to a bacterial pathogen for the first time, a number of parameters must be considered to obtain reproducible identification of mutants attenuated in virulence from different animals inoculated with the same pool of mutants.

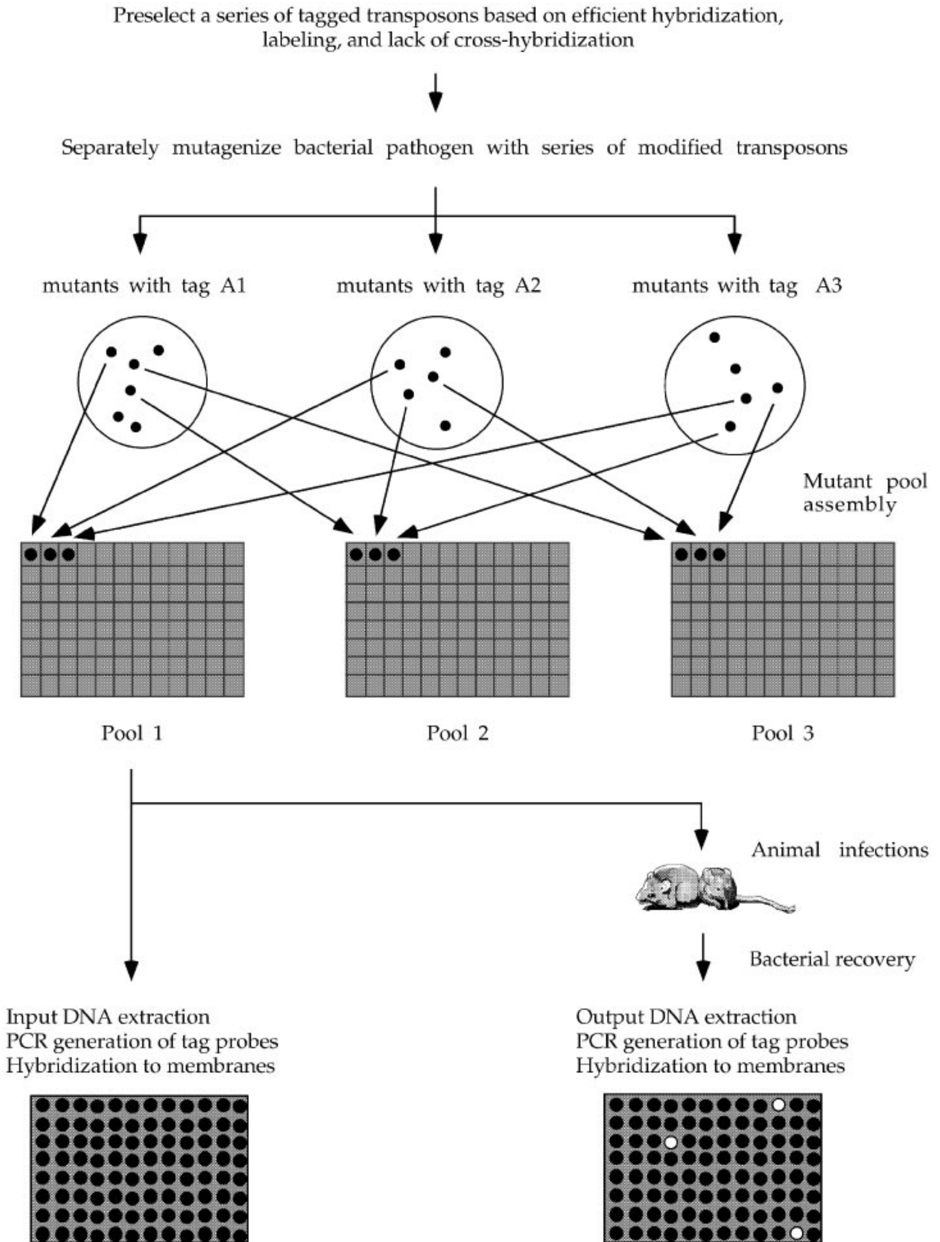


Figure 5 Schematic representation of the revised STM strategy.

Pool Complexity

As the complexity of the pool (the number of different mutant strains) increases, so must the probability that virulent mutants will fail to be recovered in sufficient numbers to yield hybridization signals, and this could lead to false identification of attenuated mutants. For *S. typhimurium* inoculated into mice by the intraperitoneal route, pools of 96 different mutants gave reproducible hybridization signals, whereas pools of 192 did not (32). With *V. cholerae*, even pools of 96 different strains did not give reproducible results, and it was necessary to reduce the pool complexity to 48 strains (17).

Inoculum Dose

If the inoculum dose is too low, there may be insufficient cells of any one virulent mutant to initiate a successful infection. For instance, a given input containing two differentially marked, wild-type strains can yield markedly different output ratios of the two strains after an infection cycle initiated by a small inoculum. Such events are reflective of a "bottleneck" in the infection process that selects individual cells stochastically that then grow out as the infection proceeds. On the other hand, if the dose is too high, the animal's immune defenses may be overwhelmed, resulting in the growth of mutant strains that would otherwise be attenuated. In *S. typhimurium*, it was found that, with a pool of 96 different mutants, an inoculum of 10^4 cells ($\sim$100 cells/mutant) gave variable hybridization patterns from animal to animal (DWH, unpublished observations), whereas an inoculum of 10^5 cells ($\sim$1000 cells/mutant) gave reproducible hybridization patterns and an attenuated virulence frequency of $\sim$4% (32). These results are consistent with studies of *S. typhimurium* and *S. paratyphi* in mice, which showed (more than 30 years ago) that bacterial cells cause infection by independent rather than synergistic action (47).

Route of Inoculum Administration

The route of administration of bacterial inoculum also influences the numbers of bacterial strains that reach the target organ(s) and tissues, hence the reproducibility of tag hybridization signals. For example, if inoculated by the intraperitoneal route, 10^5 *S. typhimurium* cells representing a pool of 96 mutants yield reproducible hybridization signals for the vast majority of strains recovered from the spleens of infected animals. If the same inoculum is given orally, however, only a small percentage of mutants are subsequently found in the spleens, and the identity of these varies from animal to animal (J Shea, DW Holden, unpublished observations). Evidently, the majority of cells in the inoculum fail to cross the gut epithelium, either because they are rapidly cleared from the small intestine or because the M cells of Peyer's patches, through which the majority of bacteria are thought to gain access to the deeper tissues of the host (19, 20, 62), represent an infection bottleneck, and only a relatively small number of bacteria proceed to cause systemic disease. These observations suggest that, apart from its use in

studies of bacterial virulence, STM might also prove to be useful in studies of the population dynamics of virulent strains during the course of infection. These types of studies have hitherto been restricted by the small number of markers available for strain identification (47, 52).

Duration of Infection

Another important aspect of the STM screening process concerns the postinoculation time point at which bacteria are recovered to prepare tags for hybridization analysis. If this time period is short, virulent cells may have had insufficient time to outgrow the attenuated strains to a degree that is reflected in a clear difference in hybridization signal intensity of tags on the blots. On the other hand, if the period is too long, there may be a risk that some virulent strains may simply outgrow other virulent strains in a nonspecific manner.

The parameters described above are obviously interrelated and must be optimized empirically for each pathogen-host interaction, to obtain reproducible hybridization patterns with tags recovered from at least two animals infected with the same pool of mutants.

From IVET, STM, and earlier studies (47), it is clear that, if the inoculum dose is sufficiently high, systemic *S. typhimurium* infection of the mouse involves multiplication of many of the cells present in the inoculum, rather than clonal expansion from one or a small number of cells in the inoculum. By comparing the results of STM with results from virulence tests with individual mutants at lower doses (11), it is possible to determine whether trans-complementation of mutant defects by virulent mutant strains occurs to a significant degree and whether inoculation with a mutant pool at a dose several orders of magnitude higher than the wild-type LD_{50} (<10 cells by the intraperitoneal route) overwhelms the immune response and results in the growth of strains that would otherwise be attenuated. The virulence of 330 individual Mud*J* transposon mutants was tested by intraperitoneal inoculations at a dose of 10^3 bacteria, and it was found that 1.2% had LD_{50}s >1000-fold higher than that of the parental strain (11). In the initial STM screen using mTn*5* mutagenesis of the same *S. typhimurium* strain in the same mouse strain, 3.4% of 1152 mutants were identified as attenuated, and the LD_{50}s of >70% of these strains are >1000-fold higher than that of the parental strain. There is therefore no evidence from the *S. typhimurium*-mouse interaction that mixed infections of virulent and attenuated strains inoculated at high dosages lead to a lower level of attenuated-mutant recovery than would be observed with single-strain infections at a lower dose.

The original application of STM in *S. typhimurium* (32) by intraperitoneal inoculation of mice resulted in the identification of a new pathogenicity island, SPI2, containing at least 31 genes predicted to encode proteins of a type III secretion system that is specific to the salmonellae (33, 60). Genes in SPI2 were independently identified by a genome comparison approach (54) and by DFI (67), and the SPI2 type III secretion system appears to be required for replication of bacterial cells in macrophages (18, 33, 54). Two of the SPI2 mutants were inoculated

by the peroral route and were shown to be severely attenuated as evidenced by significantly increased LD_{50} values (60). This result, along with the recovery of known virulence factors by STM (32), shows that although *Salmonella* infections are not acquired intraperitoneally in nature, this route of inoculation does provide information relevant to natural infection. By the same token, it is not surprising that genes important for survival in the gut and for translocation across the gut epithelium (28) were not identified by STM screening.

Virulence in *Staphylococcus aureus* has been studied by using the modified STM methodology described above (22, 45, 59). In the study by Mei et al (45), Tn*917* mutants were tested in a murine model of bacteremia. The majority of loci from 50 mutants that were identified as attenuated were predicted by sequence similarity to be involved in cell surface metabolism (e.g. peptidoglycan cross-linking and transport functions), nutrient biosynthesis, and cellular repair processes, but most of the remainder had no known function. A slightly larger signature-tagged mutant bank was constructed by using the same transposon and tested in models of bacteremia, abscess and wound formation, and endocarditis (22). This enabled the identification of various genes affecting growth and virulence in specific disease states, as well as 18 that are important in at least three of the infection models. Many of these genes appear to be involved in the same kinds of processes as those identified in the earlier study (45); indeed, seven of the genes identified by Mei et al (45) were also found by Coulter et al (22).

STM was also used to isolate colonization-defective mutants of *V. cholerae* (17), and the screen resulted in the identification of a number of genetic loci critical for colonization of the infant mouse intestine. As expected, several of these genes were previously known to be involved in biogenesis of the toxin coregulated pilus, which is absolutely required for efficient colonization in both infant mice and humans (6, 34, 36, 64, 65). Mutations in purine, biotin, and lipopolysaccharide biosynthetic genes were also found to cause severe colonization defects. Two loci identified by STM appear to encode phosphotransferases, and mutations in these genes affect coordinate regulation of virulence factors in *V. cholerae*. Other identified loci had no previously known function in pathogenesis, and one had no homology to any known genes.

A further modification of the basic STM method involves hybridization of tags to high-density arrays, in an approach termed molecular bar coding (61). Its potential feasibility was demonstrated in a pilot study with 11 auxotrophic *Saccharomyces cerevisiae* deletion strains to monitor the depletion of some of these strains in media lacking the relevant metabolite. Molecular bar coding appears to be quantitative, and it may be particularly useful for studying mutant strains with subtle phenotypic defects. It may also be capable of processing very large numbers of strains simultaneously because the tag population is monitored by hybridization to a high-density oligonucleotide array, but it should be noted that, although this could potentially permit simultaneous analysis of thousands of strains, pool complexity would still be subject to biological constraints such as in vivo bottlenecks.

ADVANTAGES AND DISADVANTAGES OF IVET AND STM

The studies described above demonstrate that IVET is quite capable of finding novel virulence genes, although the rate of success rather depends on the definition of virulence gene. Not all *ivi* mutations result in pronounced virulence defects as evidenced by vastly increased LD_{50} values or complete inability to survive in the host, but many do cause decreased ability to persist in host tissues. They could also be responsible for damage to the host, which has not been assessed in most IVET studies. It may be that many *ivi* loci make small individual contributions to virulence, and their effects may be additive or synergistic. There have been no published studies examining the effects of multiple *ivi* mutations in a single strain, presumably owing to technical considerations, but this could be a fruitful approach eventually.

The most significant disadvantage of IVET is that, in most of its current incarnations, it discriminates perhaps too strongly against genes that are expressed in vitro. These are almost invariably removed from the pool at some point, although there is no reason to expect that in vitro-expressed genes would not be important for either survival in the host or to cause damage to the host.

With IVET methods, it is necessary to bear in mind that the in vitro conditions may have a profound influence on the nature of the genes isolated. For example, if essential biosynthetic genes are induced in response to the lack of a particular nutrient, then the presence or absence of that nutrient in the in vitro situation may determine whether these biosynthetic genes are identified by IVET as host inducible. Growth on minimal media would cause such genes to be expressed in vitro, which in turn would lead to their elimination from consideration. On rich media, however, the genes might be expressed at a low level, and they would be identified as *ivi* loci if their expression were subsequently induced in the nutrient-limited host. A similar argument applies to any gene, and because many virulence genes are already known to be regulated by environmental signals, the choice of in vitro conditions becomes a major consideration when using IVET to search for virulence factors.

To date, no published IVET strategy has attempted to identify genes whose expression must be downregulated during infection, although this could be a valuable approach. In *Bordetella bronchiseptica*, for instance, it appears that flagella are not produced during infection in rat and rabbit models, and forced expression of flagella during rat infection in fact results in decreased colonization (1). Although flagella are not required for successful infection in rats and so perhaps would not commonly be described as virulence factors, the knowledge that ectopic production of flagella reduces colonization surely increases our understanding of the infectious process.

The preceding discussion makes it clear that, although most current IVET methods aim to detect increases in promoter activity, ideally IVET should be capable of studying both increases and decreases in promoter activity. It would also be desirable to be able to quantitate such changes in expression level. Studies of this

nature may be possible with antibiotic IVET selections, which theoretically permit identification of *ivi* promoters with different levels of activity through variation of antibiotic levels and administration. No such studies have yet been carried out, and it has not been determined whether the levels of antibiotic can be controlled at a sufficiently fine level both in vitro and in vivo for this method to be implemented easily. However, DFI provides a simpler way to accomplish the same goals, because *gfp* fusions are not required for survival during infection and strains carrying such fusions can be efficiently and arbitrarily sorted by their green fluorescent protein activity. Even more exciting is the finding that active *gfp* fusions can be detected by FACS analysis of homogenized tissue from infected animals (67). This indicates that DFI is useful not only in cell culture models of virulence, but that it might also be used to isolate active fusions directly from animals.

Every strain identified by STM is by definition attenuated for survival under the specified conditions, regardless of the expression patterns of the gene mutated in that strain. It is therefore a more direct method than IVET for isolating genes required for survival in the host, because genes identified by IVET must be mutated subsequently to demonstrate their requirement for virulence. On the other hand, STM does not select positively for mutants bearing the desired traits. The host animal selects against the interesting mutants, but these can be identified only postinfection by hybridization screening. Therefore, although STM is generally much less laborious than traditional "one-mutant, one-animal" screens because of its ability to screen mutants in pools, STM is not as straightforward a selection method as IVET.

The majority of mutant strains identified by STM in *S. typhimurium*, *S. aureus*, and *V. cholerae* have subsequently been shown by LD_{50} tests or competition analysis to be important for growth in vivo. Very occasionally, however, strains have been isolated with weak output hybridization signals but for which no apparent virulence defect could be demonstrated (R Mundy & DW Holden, unpublished observations). The reason for this is not known.

Not surprisingly, only a subset of the genes identified by IVET as host-induced were found to have a substantial role in virulence as assessed by LD_{50} or competition assays. In *S. typhimurium*, IVET identified previously known virulence genes (such as *phoP*) and several novel genes whose inactivation did not produce a noticeable virulence defect in LD_{50} assays (31). Of 11 *S. aureus ivi* genes that were mutated, 7 of the corresponding mutant strains had a virulence defect (40). In *V. cholerae*, 3 of the 13 identified *ivi* genes had a demonstrable role in colonization (15).

It is curious that, whereas many of the genes identified by IVET and STM in *S. typhimurium* are clearly "virulence determinants" in the classical sense of the term, many of the *S. aureus* genes identified by IVET and STM appear to have more fundamental roles in bacterial metabolism. Although IVET successfully identified the *S. aureus* virulence factor *agrA*, genes for other known virulence determinants, such as toxins and extracellular matrix-binding proteins (41), have not yet been identified by these screens (22, 40, 45). There are several possible explanations for this. First, it may be that these factors are expressed at too high

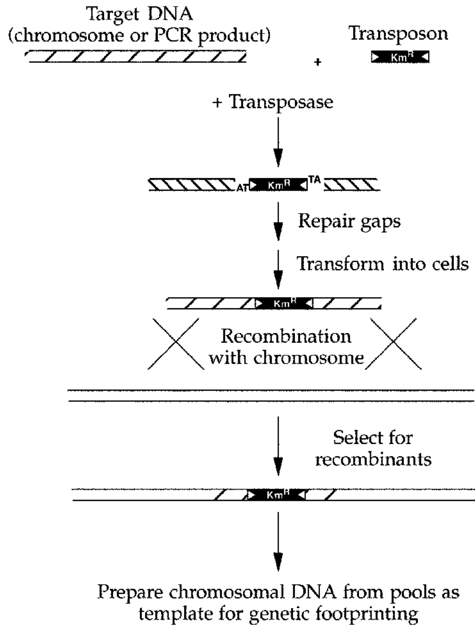
a level in vitro to qualify as in vivo-induced. Second, for STM, transposon mutagenesis is not fully random and may have favored mutation of certain areas of the chromosome over others. Third, mutation of genes encoding toxins may result in trans-complementable phenotypes. Fourth, no STM screen to date has examined more than ~1500 different mutants, so the screens are probably not saturating. It should also be noted that, depending on the sensitivity of the PCR/hybridization protocol for detecting such changes in tag populations, STM might not identify mutations causing small or even moderate reductions in survival. This is a particularly important consideration because this category includes mutations in genes that are critical for causing disease but do not appreciably affect survival of the bacterium in the host. For example, it is the action of cholera toxin that is primarily responsible for the lethality of cholera (9), but deletion of the cholera toxin genes does not affect the bacterium's ability to colonize the host (64).

The types of genes that could in theory be identified by IVET and STM can be summarized as follows: STM should identify a subset of genes that are required for growth in vivo; IVET should identify some genes that are required for growth in vivo and others that are not, because not all genes that are expressed in vivo are required for survival in vivo, and some genes that are required for growth in vivo may also be expressed in vitro; STM would not be expected to identify genes that are essential for bacterial growth, nor would IVET unless the genes were expressed at a sufficiently low level in vitro. The results of the IVET and STM studies reported to date support these predictions. Moreover, based on the studies in *S. typhimurium*, *S. aureus*, and *V. cholerae*, there seems to be little overlap between the genes identified by IVET and STM, so these two approaches appear to be genuinely complementary.

GAMBIT

Essential genes are by definition required for growth or viability in vitro. Because one would expect such genes to be expressed under all conditions, they would not be identified by most IVET methods unless they were expressed at extremely low levels in vitro. This particular category of *ivi* genes may have been documented by Lowe et al (40) in that they identified several *S. aureus* genes that were in vivo-induced but that could not be disrupted in subsequent analyses. STM is equally unable to assess the role of essential genes in pathogenesis, because transposon insertions in these loci would be expected almost invariably to be lethal. This is a critical issue because essential genes are prime targets for antimicrobial strategies. For these reasons, a systematic and efficient means of studying essential genes is certain to contribute greatly to our understanding of pathogenic processes.

The recently developed GAMBIT method was designed to identify essential genes in naturally transformable organisms whose genomes have been sequenced (2). The name GAMBIT stands for "genomic analysis and mapping by in vitro transposition," and the procedure is outlined in Figure 6. A specific region of the chromosome is amplified by extended-length PCR, and the product is subjected to



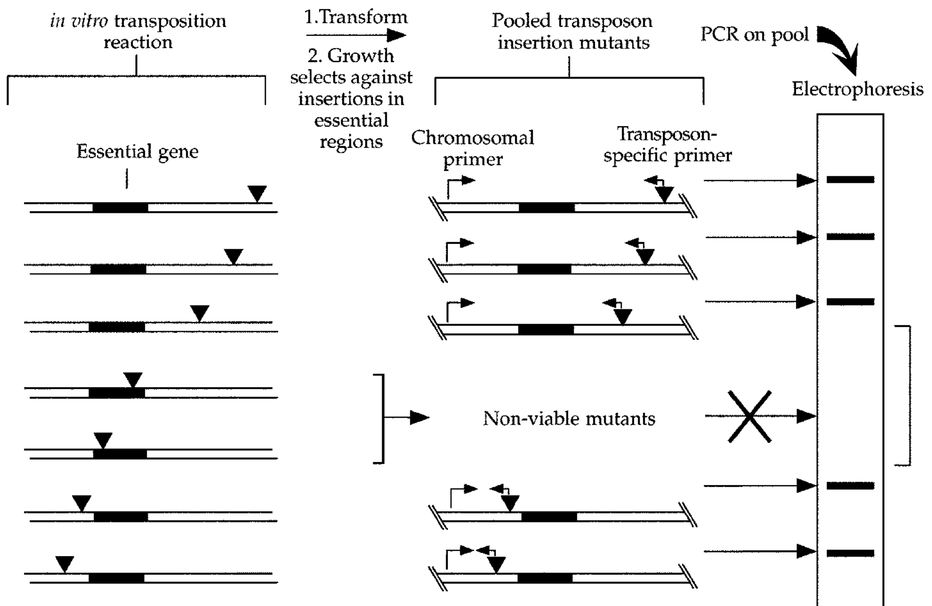


Figure 6 Schematic diagram of the two steps required for GAMBIT. (*a*) Strategy for production of chromosomal mutations by in vitro transposon mutagenesis. (*b*) Genetic footprinting for detection of essential genes. Reprinted from Reference 2.

in vitro transposon mutagenesis. The resultant pool of mutagenized DNA is then transformed into bacteria, which are then grown under selective conditions (e.g. on defined medium or in an animal). PCR is then performed on the postselection pool using a transposon-specific primer and a primer to a known location on the chromosome. Subsequent analysis of the PCR products allows determination of which genes in that region of the chromosome are required for survival under those selective conditions. This type of PCR analysis of transposon insertions has been termed "genetic footprinting" and was first tested in *Saccharomyces cerevisiae* (63).

The ability of GAMBIT to identify in vitro essential genes was confirmed in both *Haemophilus influenzae* and *Streptococcus pneumoniae* (2), and GAMBIT has already been applied to the problem of identifying genes essential for growth of *H. influenzae* in a mouse model (3). Like STM, the use of GAMBIT in animal models constitutes a negative selection in which certain mutants are eliminated by selection in the animal. These mutants are recognized by the loss of PCR products corresponding to insertions in the in vivo essential genes that are represented in the preinfection inoculum. A particularly attractive aspect of GAMBIT analysis is its ability to target specific genes or regions of the chromosome. Although it is necessary to design quite a large number of PCR primers ($\sim$130 primers per Mb of target DNA) to apply GAMBIT to entire genomes, the facility with which this method defines essential genes should make it enormously useful in both the study of microbial pathogenesis and the development of antimicrobial drugs. Finally, the development of efficient DNA transformation methods should enable the adaptation of this system for the analysis of bacteria that are not naturally competent.

CONCLUDING REMARKS

In recent years, sequences of entire bacterial genomes have been obtained with greater rapidity and ease than thought possible only a short time ago, and this is revolutionizing our understanding of and experimental approaches to the study of bacterial virulence. With good annotation, genomic sequences will constitute a powerful genetic "infrastructure" capable of providing not only the sequence of all of an organism's genes but also functional information for some of them. It is nevertheless clear that, for the majority of genes, it is not possible to determine the biochemical function of their products from their DNA sequences. Therefore, a continuing need exists for gene expression and mutational studies to provide phenotypes that can be used to characterize the functions of these genes. Such studies are also necessary for genes with known function that were not previously suspected to play a role in pathogenesis.

The value of the IVET and STM methods is that they allow these types of analysis to be performed simultaneously on a relatively large number of genes during an actual infection. STM is most useful for determining outright which loci contribute

strongly to survival in the host, whereas IVET strategies are capable of providing more subtle information regarding the expression patterns of genes during infection. Comparison of the IVET and STM results in *S. typhimurium*, *S. aureus*, and *V. cholerae* shows that they are complementary approaches. The integrated use of these approaches is already well underway in a variety of organisms, because current work in this field includes STM analyses in *Streptococcus pneumoniae*, *P. aeruginosa*, *Y. enterocolitica*, and *Legionella pneumophila* and the application of DFI in *L. pneumophila* and *Bartonella henselae*. The information obtained from such studies will undoubtedly contribute to a more comprehensive understanding of bacterial pathogenesis.

ACKNOWLEDGMENTS

Work in the Holden laboratory was supported by the MRC (U.K.), Wellcome Trust (U.K.), Pharmacia and Upjohn, Inc., and SmithKline Beecham plc. Work in the Mekalanos laboratory was supported by National Institutes of Health grants AI18045 and AI26289 (to JJM).

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