Figure 1. The authors have also carried out X-ray structural analysis of the receptor, creatinine, and the complex to demonstrate some of the finer details of the binding.

The authors have shown that this synthetic receptor is selective for creatinine, since no colour change was observed with creatine, urea, uric acid, proline or histidine. The estimated dissociation constant of the complex (0.5 µM) is also sufficiently low so that blood serum creatinine (typically 40–130 µM) can be sensed by it. Alkali metal ions were found to give some chromogenic response with the receptor, but those were significantly weaker. The prospects for the use of synthetic receptors in medical diagnostics are bright.


Uday Maitra is in the Department of Organic Chemistry, Indian Institute of Science, Bangalore 560 012, India

Pathogenesis: Generating new paths in understanding bacterial virulence

Kakoli Mukhopadhyay

The survival strategy of bacterial pathogens requires establishment in a suitable niche within another living organism, outwitting it by avoiding its formidable immune system, acquiring nutrients from it and multiplying, thus causing disease in the host. The host–pathogen interaction is a battlefield where the infecting organism encounters a range of adverse environmental conditions. These, along with other selective pressures, appear to have directed the evolution of specialized regulatory systems controlling the expression of virulence factors in the pathogen. To understand mechanisms by which the bacterial parasites circumvent or avoid the immune system of the host and cause disease, it is necessary to identify and characterize the bacterial genes and gene products responsible for the virulence at each stage of the development and progression of the disease. Traditionally, the strategies for identification of virulence factors included the three following approaches:

1. Brute force screens, that pinpoint the virulence genes on the basis of mutational loss of a virulence phenotype.2
2. Cloning screens, that pick up novel virulence genes depending on their ability to confer a virulence-associated phenotype in a heterologous bacterium3.
3. Regulatory screens, that identify potential virulence genes by their coordinate expression with other known virulence genes under defined laboratory conditions.4

All these strategies, carried out in vitro were, however, very often limited by their inability to reproduce accurately the complex and changing environments encountered by the pathogens in their hosts. Moreover, there could also be certain factors which would be induced only under specific environments within a host and could never be expressed, or were expressed in extremely low and negligible amounts, in vitro. Therefore it remained quite possible for one to miss out these genes if only the traditional methods were followed.

To overcome these limitations, Mahan et al.5 developed, in 1993, a novel genetic system, which they termed 'in vivo expression technology' (IVET). What made this strategy unique was their use of simple laboratory techniques to randomly choose genes (or only their promoters) from virulent pathogens, induce their expression within the host (i.e. in vivo), and then compare this with their expression in vitro. This then allowed them to select for those genes which were transcribed better or exclusively so only in the presence of the host environment. This, in fact, was the first attempt to study the induction of pathogenesis-related genes in the actual host environment conducive to disease development.

The first step in the technique consisted of the selection of a bacterial cell with a mutation in a biosynthetic pathway gene that greatly attenuated growth in vivo. For this purpose, Mahan et al. used a purine auxotroph (purA) of Salmonella typhimurium. Growth of such a strain in vivo was possible only if a wild-type purA gene could be introduced into it. A plasmid (pIVET) was constructed by inserting a fusion of the wild-type purA gene with a marker gene (in this case, lacZ) into a broad host-range suicide vector (pCP704). The chromosomal DNA of S. typhimurium was fragmented by a specific restriction enzyme and cloned into the plasmid pIVET, 5' to the purA-lacZ fusion, thus giving rise to a transcriptional fusion where a S. typhimurium promoter would drive the transcription of a wild-type purA-lacZ fusion. A pool of pIVET containing these transcriptional fusion constructs was introduced into purine-deficient S. typhimurium cells.
Selection for ampicillin-resistant bacterial cells now enabled the identification of those in which the pIVET1 had integrated into the bacterial chromosome by homologous recombination with the cloned S. typhimurium DNA present on the plasmid (Figure 1). Thus, in these cells, the level of purA expression required to supplement the parental purine auxotrophy should logically correspond to the level of lacZ expression that would, in turn, give a Lac⁺ phenotype. Only 33% of the cells grown in vitro showed Lac⁺ and the corresponding PurA⁺ phenotype. A pool of these recombinant bacteria were injected intraperitoneally into a BALB/c mouse. The surviving bacterial cells were isolated from the spleen after three days and this procedure repeated once more. The cells that could overcome the purine deficiency due to the presence of the integrated purA gene were expected to survive and multiply in the mouse. The cells recovered from the spleen indeed showed an increase in their number (86%) compared to that in the initial inoculum. However, there were also a group of cells, though very small in number (5%), which showed a Lac⁻ phenotype in artificial medium. The question that naturally arises here is how could these cells manage to survive in spite of a very low probability of doing so. A reasonable explanation could be that the promoter that drove the purA-lacZ fusion gene in these cells could be turned on only in vivo and not under laboratory conditions. Therefore, the genes that were regulated by these promoters under natural conditions were aptly termed as 'in vivo-induced' (ivi) genes. This phenomenon was confirmed by assaying β-galactosidase activity in the bacterial cells recovered from the spleen and comparing it with that of the same strain grown overnight in the laboratory, on enriched medium. In all the cases tested, the activity was actually 40 to 1000-fold greater in the cells grown in vivo.

The next step included the selection, cloning and sequencing of some of the ivi genes identified by the above technique. Sequence analysis could classify 15 of these genes into 5 different classes. Two of these turned out to be previously unknown genes with no homology to sequences in an established gene bank. These genes had probably not been identified earlier due to their inability to express in vitro. The other 4 genes included a carAB operon, a pheST himA operon and a rfb operon. The former consists of genes coding for two subunits of carbamoylphosphate synthase, an enzyme involved in arginine and pyrimidine biosynthesis. The low availability of pyrimidine in the animal tissue was obviously the force driving the induction of these genes. The IVET, in this case at least, helped in the identification of genes involved in intermediary metabolism and whose induction appeared to be necessary for the survival of the pathogen in the host tissues. The pheST himA operon is known to encode two subunits of phenylalanine tRNA synthetase and one subunit of the integration host factor (IHF). The latter is a DNA-binding protein required for essential functions like DNA replication, gene regulation and site-specific DNA recombination. The IHF has also
been implicated in regulating the expression of type I pilin (a protein conferring on S. typhimurium the ability to prevent clearance from animal tissues). A third fusion, the rfb operon, encodes approximately 20 genes involved in the synthesis of O-antigen (the outermost layer of lipopolysaccharide of S. typhimurium).

The ultimate goal of the experiments done by Mahan et al. was to identify the ivi genes involved in pathogenesis, which were otherwise not being picked up by the traditional techniques. Mutant strains, defective in ivi expression were then constructed to test their overall contribution to pathogenesis. These mutants, as expected, showed significantly less expression of the lacZ gene. This was also accompanied by a marked decrease in virulence as observed by a 2×10^3-fold increase in the lethal dose. Moreover, virulence was so diminished that mice could even be immunized with these mutant strains and rendered resistant to infection by S. typhimurium, administered orally.

Many other pathogenic bacteria are known to be attenuated by purine auxotrophy.7-11 This technology can therefore be useful in studying a variety of host–pathogen systems. Various other biosynthetic genes can also be used for this selection scheme. One such example is a thyA–lacZ fusion construct which could be used in bacterial strains with thymine auxotrophy resulting in their attenuation. The advantage of this system is the easy selectability of thyA mutants with trimethoprim.

A drawback of the IVET was its applicability only in host–pathogen systems where attenuating auxotrophies prevailed. However, earlier this year, Mahan et al. expanded the scope of this technology by developing a transcriptional fusion vector using antibiotic resistance as the basis for selection in host tissues.12 This new vector contains a fusion of promoterless chloramphenicol acetyltransferase (cat) and lacZ genes. BALB/c mice were infected with a pool of S. typhimurium clones carrying random cat–lac transcriptional fusions and were subsequently given intraperitoneal injections of chloramphenicol. The strains which survived the passage through the animals expressed the cat gene and were then screened for those that had low-level lacZ expression on laboratory medium. These strains would carry fusion constructs containing specific ivi genes. A major advantage of the antibiotic-based IVET selection is that it does not require the isolation and characterization of an attenuated mutant strain with a genetically defined nutritionally-deficient species as in the original IVET protocol. Thus, the antibiotic-based IVET selection should also be applicable to fastidious organisms where genetic markers, suitable for nutritional IVET schemes, are not readily available. Subtractive hybridization could be one way of identifying or estimating the expression of ivi genes of these microorganisms.13 But the extremely low quantity of bacterial RNA produced in the host tissues could pose a limitation in achieving this.

One of the most challenging environments that invading bacteria encounter within the host is the host macrophages which eliminate the pathogens in the majority of cases. S. typhimurium, however, is an exception in that it has evolved mechanisms to survive within the inhospitable environment of the phagolysosomes. Genes of this organism, which are specifically induced in cultured macrophages, could also be isolated by the IVET. Characterization of the ivi genes of S. typhimurium in macrophages would help in tracing the unique mechanism by which this pathogenic species survives the unfavourable host environment.

With the introduction of antibiotic-based selection of ivi genes, the IVET can be extended to a broader host/host-pathogen range, thus opening up the possibility of studying a larger number of genes involved in various metabolic pathways in greater detail. Such studies may give us an insight into the underlying mechanisms regulating the development of different diseases and thus provide clues for the design of specific drugs against them.

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Kakoli Mukhopadhyay is in the Department of Crop Physiology, University of Agricultural Sciences, GKVK, Bangalore 560 065, India.