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Genomic approaches to understanding bacterial virulence

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The genomic sequences of bacterial pathogens and of the host species they infect have greatly increased the understanding of host–pathogen interactions. Sequences of bacterial genomes have led to the identification of virulence factors through the use of bioinformatics, targeted mutant library construction, screening approaches combining transposon mutagenesis and microarray technology, and through the expression of libraries of bacterial proteins within model organisms such as yeast. Host genomic information has also yielded insights into bacterial virulence through transcriptional profiling of host responses to infection and identification of host proteins required for bacterial pathogenicity using knockdown of host gene product expression during infection. Research using genomic approaches to bacterial pathogenesis is a rapidly growing field and will expand further as additional bacterial genome sequences become available and techniques for conducting high-throughput analysis are refined.

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Introduction

The genomes of many bacterial pathogens have now been sequenced. These sequences, combined with genomic sequences of human and model organisms, have led to a dramatic increase in the use of genomic approaches for studying bacterial virulence (for reviews see [1•,2,3]). Here, the focus is on genomic approaches that have been recently developed and/or refined that use bacterial and host genome sequences. Bacterial genome-based approaches discussed here include bioinformatic methods, enhanced mutant library construction, genome-wide analysis of bacterial transcription factor binding, and expression of putative bacterial virulence factors in yeast. Genomic approaches have also been used to profile host gene expression changes following infection. Additionally, knockdown of expression of host gene products by using RNA interference (RNAi) or antisense-mediated knockdown during infection have become powerful methods to identify host factors required for bacterial pathogenicity.

Bacterial genome-based approaches

Bioinformatic approaches

For many bacterial pathogens, genomes of closely related species and/or multiple strains have recently been sequenced. This has permitted comparative genomic techniques for identification of conserved genes among multiple pathogenic strains or genes that have predicted functions similar to known virulence factors [4]. Another method, incorporating both bacterial and host sequence information, is the identification of putative virulence determinants by identifying bacterial proteins with homology to eukaryotic factors [5]. For a more complete review of these techniques, see Raskin *et al.* [1•].

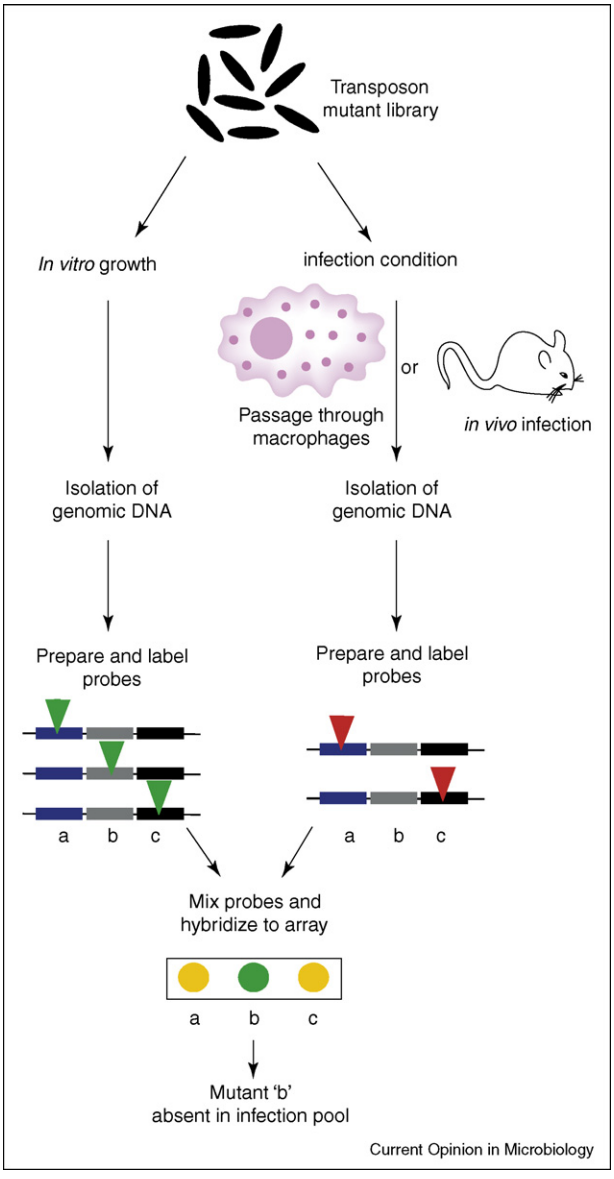
Enhanced mutant library construction and mutant identification

Knowledge of bacterial genome sequences has enabled researchers to construct complete and ordered mutant libraries of several species including *Pseudomonas aeruginosa* and *Escherichia coli* [6]. Ordered *P. aeruginosa* transposon mutant libraries with characterized insertion sites that approach saturation have been constructed in the commonly used laboratory strain PAO1 as well as in a clinical isolate, PA14 [7,8]. Mori and co-workers [9] have used recombination of PCR fragments into the genome of *E. coli* to construct in-frame deletions in 3985 of 4288 targeted genes and have assembled the first complete deletion mutant set for a bacterial species.

In addition to defined libraries of bacterial mutants, several groups have developed techniques using microarray technology to detect diverse transposon insertions within pooled populations. In this approach, the transposon insertion sites present in the library are identified using genomic DNA microarrays. The composition of the input pool is compared to that of an output pool and mutants that fail to survive a given condition are identified (Figure 1).

This technique has been especially useful in identifying pathogenic determinants of *Mycobacterium tuberculosis*. Sassetti *et al.* [10] used a modified mariner transposon to construct a mutant library of *M. tuberculosis* strain H37Rv and subsequently identified bacterial genes required for infection in mice. One library pool was grown *in vitro* and the other was isolated from the spleens of infected mice, and transposon mutants that were missing

Figure 1



Screening method for microarray identification of negatively-selected transposon mutants. Pools of transposon mutants are used in a specific infection condition, such as growth in macrophages or persistence during infection of an animal, whilst control pools are grown *in vitro*. Genomic DNA is isolated from each pool and fluorescently labeled probes corresponding to genomic regions immediately flanking the transposon insertions are prepared. The relative prevalence of a given mutant *in vitro* over that in the infection condition is then determined by competitive hybridization to a genomic microarray. Modified from [11] (Copyright 2005 National Academy of Sciences, USA).

from the *in vivo* infection condition were identified. The transposon construct that was used enabled determination of insertion sites by a combination of *in vitro* transcription, initiating from an internal T7 promoter, and PCR followed by fluorescent labeling and hybridization to a genomic microarray [10]. This approach is referred to as transposon site hybridization (TraSH). A subsequent study expanded upon the *in vivo* mouse studies and identified transposon mutants that failed to survive during prolonged infection of murine macrophages [11]. Macrophages are the primary niche for *M. tuberculosis* replication *in vivo* and are reservoirs during latent *M. tuberculosis* infection. The results of the two screens were compared in order to identify genes required for survival both in macrophages and in infection *in vivo*, those required for growth in macrophages only, or for systemic infection *in vivo* [10,11]. Interestingly, several phosphate transport genes including *phoT* were identified as being essential for growth in macrophages, but not for splenic infection [11].

Bishai and co-workers [12] have modified the above screening approach in order to identify *M. tuberculosis* genes required for virulence by collecting defined transposon mutants and constructing arrays designed to identify the presence or absence of each mutant. They have named this approach DeADMAn (designer arrays for defined mutant analysis) and have applied the technique to identify genes required for survival of *M. tuberculosis* in mouse lungs. Although the scope of this approach is less than other genome-wide approaches, DeADMAn has the advantage of being archival, which enables easy isolation of the mutant of interest.

Similar approaches have also been used to identify *Salmonella enterica* serovar Typhimurium genes required for pathogenesis. Chan *et al.* [13] constructed a mini-Tn10-based transposon library in *S. enterica* serovar Typhimurium and identified transposon mutants negatively selected during infection of murine macrophages and mutants that failed to colonize the spleens of BALB/c mice. Many genes from SPI2 (*Salmonella* pathogenicity island 2) were identified as essential for infection of the macrophage cell line as well as *in vivo* infection of BALB/c mice. This observation is consistent with known roles for SPI2 virulence genes in the establishment and maintenance of the specialized intracellular replication niche, the *Salmonella*-containing vacuole. Intriguingly, 22 genes from SPI1 (*Salmonella* pathogenicity island 1) were identified in the macrophage survival screen. This was unexpected because SPI1 genes have been primarily associated with invasion of cells in the gastrointestinal track and have not been shown to be important for survival in macrophages [13]. The transposon library was also used to identify *Salmonella* genes required for persistent systemic infection in mice [14•]. Several commonly used laboratory strains of mice, including BALB/c, lack a functional version of the Nramp1 protein, which aids in the control of *Salmonella* replication. Infection of mice harboring a functional Nramp1 protein typically results in a persistent systemic infection rather than a lethal infection. Lawley *et al.* [14•] infected 129X1/SvJ mice containing functional Nramp1 with the transposon

library, followed by microarray comparison of input and output pools, in order to identify genes required for long-term systemic infection. Interestingly, over 50% of the factors identified corresponded to genes with putative or unknown functions. Similar to the results in the macrophage screen and the acute infection screen in BALB/c mice, transposon mutants in SPI1 and SPI2 genes were negatively selected during long-term infection. The negative-selection of SPI1 genes following intraperitoneal infection, together with the macrophage screen results, indicate that SPI1 might indeed have roles beyond the gastrointestinal phase of infection [13,14•].

Genome-wide location analysis of transcription factor binding

Transcriptional profiling using microarrays to compare gene expression patterns in wild type and mutant bacterial strains has been successfully used to identify genes regulated by transcription factors or other regulatory proteins. A limitation of this approach is that indirect effects on transcription are not distinguished from direct interaction of the regulator with the promoter region of the gene of interest. One method of identifying genes directly controlled by a specific regulator is to combine transcriptional microarrays with chromatin immunoprecipitation, in which regions of DNA bound by a protein are isolated, followed by genome-wide identification of the regulator binding sites using microarrays. Whereas this approach was initially used in non-pathogenic bacterial species [15–17], genome-wide location analysis of transcription factor binding has now been used to identify gene regions directly bound and controlled by the ferric uptake regulator protein, Fur, in the human pathogen *Helicobacter pylori* [18]. Fur has important roles in *H. pylori* virulence [19], and it was found to extensively bind the *H. pylori* genome, including a significant number of genes not known to be regulated by iron levels. This work also showed that Fur acts as a positive regulator of transcription for motility and chemotaxis genes such as *flaB* that are essential for colonization of the human gastric mucosa [18]. The genome-wide location approach might be useful to identify novel virulence factors in other pathogens by examining genes that are directly regulated by known virulence regulators and might also more clearly define the complicated regulatory circuits that exist in many bacterial pathogens.

Expression systems in yeast

In pathogens harboring secretion systems designed to transfer bacterial effector proteins into host cells, determining the host targets of these effectors that contribute to virulence is of significant interest. The yeast *Saccharomyces cerevisiae* is a well-established model eukaryote and has numerous pathways that are well-conserved in higher organisms such as humans. *S. cerevisiae* has emerged as a useful tool for the characterization of bacterial effector proteins because of the availability of a large number of yeast mutants and the ease of genetic manipulation. Whereas initial studies focused on identifying the eukaryotic targets of known type III secretion system (TTSS) effector proteins from *Yersinia enterocolitica* and *S. enterica* serovar Typhimurium [20], further work has used the yeast model to identify novel effectors and additional virulence-associated proteins.

Several effectors of the *Legionella pneumophila* Dot/Icm (defective organelle transport/intracellular multiplication) type IVB secretion system (TFBSS) have been identified using expression of random *L. pneumophila* genomic DNA libraries in *S. cerevisiae*, followed by screening for *Legionella* proteins that, when overexpressed in yeast, resulted in a yeast growth defect or interfered with vacuolar protein sorting [21,22]. Campodonico *et al.* [21] identified the Dot/Icm substrate YlfA (yeast lethal factor A) by screening for *Legionella* proteins that resulted in a yeast growth defect when overexpressed in *S. cerevisiae*. Further experiments showed that YlfA protein localizes to the endoplasmic reticulum (ER)-derived *Legionella* replicative vacuole and punctate structures throughout the cells late after infection in mammalian cells [21]. Shohdy *et al.* [22] identified three VPS (vacuolar protein sorting) inhibitory proteins (Vips) with previously unknown function and showed that VipA, VipD and VipE were translocated into mammalian macrophages through the Dot/Icm TFBSS [22]. Valdivia and co-workers [23••] have taken a more defined approach to identifying virulence factors from a genetically intractable bacterial pathogen: 216 open reading frames (ORFs) from *Chlamydia trachomatis*, representing *Chlamydia*-specific or conserved hypothetical ORFs of unknown function and ORFs with homology to virulence factors in other bacterial species, were expressed in yeast under the control of a galactose-inducible promoter. Screening methods were then used to identify *C. trachomatis* proteins capable of disrupting eukaryotic cellular functions or localizing to specific organelles.

Host approaches

Expression profiling

In addition to identifying bacterial virulence factors, many researchers are now focusing on host factors that function to protect the host from infection or that are required by the bacterial pathogen for efficient infection. Transcriptional profiling studies examining host cell responses to infection have provided insights into the immune system components and signaling pathways stimulated by bacterial pathogens [24]. Other studies have characterized the contribution of specific virulence factors by comparing changes in expression patterns following infection by wild type bacteria relative to bacterial mutants. For example, Ichikawa *et al.* [25] examined the individual and combined effects on host gene expression of three *P. aeruginosa* TTSS effectors, ExoS, ExoT and ExoY. Gene expression changes consistent with the

known functions of the effectors and synergistic effects of the three effectors combined were observed. Although a great deal of information can be gained from transcriptional profiling studies, changes in gene expression do not necessarily correlate to a functional outcome during infection. Host functional studies have generally been inhibited by the greater difficulty of genetic manipulation in mammalian systems over manipulating most bacterial pathogens. Recent progress in mammalian gene expression knockdown techniques and the development of infection models in genetically tractable eukaryotes have begun to expand the approaches that can be used to examine the host factors required for bacterial virulence.

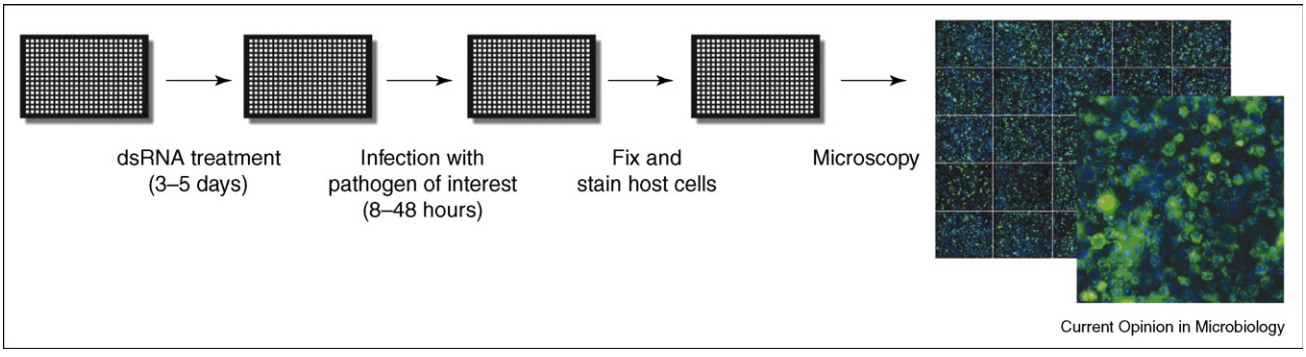
Knockdown of host gene product expression

One approach for knockdown of mammalian gene products is overexpression of antisense RNAs. Lu *et al.* [26] used antisense RNAs, encoded by a library of ~40 000 human expressed sequence tags (ESTs), to knockdown expression of human genes. Following transfection of the antisense RNA constructs, host cells were incubated with anthrax toxin protective antigen (PA) and a hybrid toxin consisting of a PA-binding domain and the ADP-ribosylation domain of *P. aeruginosa* exotoxin A. Surviving host cells with increased resistance to PA-mediated toxin entry were isolated. Knockdown of human ARAP3 — for Arf GTPase-activating protein (GAP) and Rho GAP with ankyrin repeat and PH domains, a phosphoinositide-binding protein with roles in rearrangements of the host cell cytoskeleton — was determined to confer toxin resistance [26]. These studies suggest that EST antisense libraries might be useful for other loss-of-function genetic screens in mammalian cells. Nonetheless, a caveat of antisense-based approaches is that the level of knockdown of gene expression is generally moderate. Thus, an observable phenotype must not depend on high-level knockdown efficiency.

Despite the development of EST-based knockdown approaches and recent advances in mammalian RNAi methods, genome-wide knockdown of mammalian gene expression remains technically challenging. Genome-wide RNAi libraries targeting mammalian genes have been constructed. However, because of uncertainties in whether a given RNA will cause efficient knockdown, multiple RNA fragments are included for each targeted gene, making the libraries quite large [27,28]. Nonetheless, these libraries have great promise for researchers interested in mammalian host cell factors that are required for bacterial pathogenesis, especially as smaller sub-genomic libraries targeting subsets of genes with common functional annotations are constructed and screening methodologies are improved. Meanwhile, several groups have used *Drosophila melanogaster* cells as a model for bacterial infection to conduct genome-wide and sub-genomic screens. *Drosophila* S2 cells are macrophage-like and highly amenable to RNAi technology. S2 infection models have been established for several intracellular bacterial pathogens, including *Chlamydia trachomatis*, *Listeria monocytogenes*, *Mycobacterium fortuitum* and *L. pneumophila* [29–31,32••,33•].

Genome-wide RNAi-based screens for host factors affecting intracellular infection have been completed in *Drosophila* S2 cells for *L. monocytogenes* and *M. fortuitum* (Figure 2) [32••,34••,35••]. Agaisse *et al.* [34••] and Philips *et al.* [32••] used a collection of ~21 300 dsRNAs, targeting >95% of the annotated genes in the *Drosophila* genome, to identify host factors required for *L. monocytogenes* and *M. fortuitum* infection. Cheng *et al.* [35••] used a library of 7216 dsRNAs with selection of targeted *Drosophila* genes based on homology to human and *Caenorhabditis elegans* genes. A striking feature of all three RNAi screens was the large number of genes identified with roles in host endocytic and vesicular trafficking pathways. In addition to screening for alterations of

Figure 2



Screening method for the identification of host factors required for intracellular bacterial infection using RNAi-mediated knockdown of host gene products. *Drosophila* S2 cells are incubated with dsRNAs for 3–5 days to allow for efficient knockdown of host proteins. Cells treated with dsRNA are then infected with the pathogen of interest, followed by microscopy and image analysis of infection phenotypes. Modified from [34••].

infection by wild type bacteria, Cheng *et al.* [35••] screened for knockdowns altering infection of two *L. monocytogenes* mutant strains.

As increased numbers of host factors altering the virulence of bacterial pathogens are identified, opportunities to compare and contrast the requirements of these factors by other pathogenic species will enable greater understanding of common cellular pathways subverted by multiple pathogens to enable efficient infection. One caveat to this approach is that differences in RNAi libraries and experimental procedures might lead to the identification of specific gene products in one screen, but not in another. Therefore, more fruitful insights are likely to be garnered from comparisons of genes required by multiple pathogens rather than genes that are specific for a single pathogen.

In addition to genome-wide approaches, comparisons using targeted sub-genomic libraries will provide greater insights into host pathways important for infection. The use of smaller sub-genomic RNAi libraries might be especially useful for identifying pathways that require knockdown of multiple components for a phenotype to be observed. For example, proteins related to trafficking between the ER and Golgi apparatus were targeted by dsRNA individually and in pairwise combinations and the effects on intracellular replication of *L. pneumophila* were assessed [33•]. Whereas few single RNAi targets significantly altered infection, the combinatorial approach suggested that knockdown of multiple factors within the trafficking pathways caused a stronger defect in *L. pneumophila* replication than did knockdown of single components.

Conclusions

Genomic sequences have contributed significantly to the current understanding of host–pathogen interactions, but many avenues of investigation using sequence data have not been fully explored. For example, expression of a defined subset of *C. trachomatis* ORFs in yeast led to the discovery of several putative virulence factors, implying that this technique might be useful in the identification of novel secretion system effectors and host-modulatory proteins from pathogens such as *H. pylori* and *Rickettsia* species where genetic manipulation is difficult. Similar to yeast expression studies with *S. cerevisiae* mutants and RNAi-based screens with mutants of *L. monocytogenes* [20,35••], further combinations of genome-wide approaches using bacterial and host mutants will probably be very useful in deciphering the functions of both bacterial and host gene products during infection. One of the primary challenges of genomic approaches to bacterial virulence is the integration of large quantities of data. Future developments in bioinformatic techniques for integrating data from multiple pathogens and multiple genome-wide approaches, including transcriptional and functional methodologies, will be key to fully using and comprehending the results of genome-wide studies.

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- •• of outstanding interest

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