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Genetic and genomic approaches to the analysis of *Leishmania* virulence

Stephen M. Beverley

Department of Molecular Microbiology, Washington University Medical School, 660 S. Euclid Ave., St. Louis MO 63110 USA. Telephone 314-747-2630, FAX 314-747-2634, Beverley@borcim.wustl.edu

Trypanosomatid protozoans of the genus *Leishmania* are important parasites of humans, infecting upwards of 12 million people in tropical and temperate regions of the world. These protozoans have an obligate digenetic life cycle, alternating between the flagellated promastigote form residing in the gut of the insect vector sand fly, and the intracellular amastigote stage residing within an active phagolysosome of vertebrate macrophages. How *Leishmania* carries out these developmental transitions, and the mechanisms that they employ in surviving within the host and resisting a tremendously hostile array of defenses, are key questions of interest to biologists and clinicians seeking to control these pathogens.

In the last 12 years a variety of genetic tools have been introduced that now permit manipulation of the genome of trypanosomatids including *Leishmania* with a high degree of specificity (Clayton, 1999, Swindle and Tait, 1996). These methods constitute a powerful genetic 'toolkit', allowing experimenters to take genes identified by various routes and probe their function by both gain and loss of function strategies, as well as localization using a variety of tags such as the green fluorescent protein (GFP). Complementing our ability to carry out reverse genetic manipulations has been the development of methods for 'forward' genetics and functional genetic rescue. Lastly, *Leishmania* has joined many other microbes in entering the era of genome science with a rapidly progressing genome project. When available, the complete genome sequence will provide tremendous new opportunities for gene discovery and analysis.

In this chapter I discuss genetic approaches available for the study of *Leishmania*, from the perspective of studying parasite virulence throughout the infectious cycle.

1. An introduction to *Leishmania* biology and virulence.

Leishmania are transmitted by the bite of Phlebotomine sand flies. Within the sand fly, parasites undergo a number of developmental transitions essential for survival and subsequent transmission by the time of the next bite. Initially parasites are taken up (most probably within

infected macrophages) with the blood meal, which is contained within the sand fly midgut by a peritrophic matrix (Pimenta et al., 1997). There, parasites are released from the ingested macrophage and differentiate to the dividing procyclic promastigote stage. After a few days the peritrophic matrix breaks down, possibly accelerated by the secretion of chitinase from the parasite (Schlein et al., 1991, Shakarian and Dwyer, 1998). At this point the procyclic parasites attach to the lumenal surface of the midgut, via binding to the abundant parasite surface glycolipid lipophosphoglycan (LPG) (Sacks, 2001, Pimenta et al., 1992). During this period of time parasites undergo extensive replication, however as the blood meal is digested parasite growth ceases and the parasites differentiate into the highly infective metacyclic promastigote. Metacyclics undergo changes in the structure of the LPG that reduce its ability to bind to the midgut, thereby allowing release of the parasite and freeing it for migration elsewhere in the alimentary tract in preparation for transmission to the mammalian host.

Once metacyclic parasites are inoculated into the mammalian host, they must first resist the action of serum complement, and then bind and enter macrophages. There the parasite differentiates into the amastigote stage, which is adapted for life within the active phagolysosome. During the initial period while the parasite differentiates, it transiently undergoes a period in which phagolysosome maturation is inhibited, again primarily by the action of LPG (Desjardins and Descoteaux, 1997). As the parasite matures to the amastigote form LPG synthesis is shut down and phagolysosome maturation now proceeds. In some manner, the amastigotes are adapted to life in an acidified, fusogenic phagolysosome. Amastigotes also greatly affect host cell signaling pathways, in most cases down-regulating or inactivating them (Alexander et al., 1999, Reiner, 1994). Which parasite molecule(s) and which host pathways are essential for survival are as yet poorly defined, although a number of candidates have been proposed.

The study of *Leishmania* biology is greatly aided by the availability of good *in vivo* and *in vitro* models relevant to host-parasite interactions. Inbred lines of laboratory mice have been intensively studied and show wide variation in susceptibility and pathology; some lines are good models for cutaneous disease, others are good models for visceral disease (Blackwell, 1996). This in combination with the large collection of mouse knockouts now available allows host genetics to be applied to the question of parasite virulence very productively, although this will not be discussed further here. Macrophages can be cultured *in vitro* and macrophage-like cell lines are available, facilitating the analysis of parasite survival in the relevant cell type. On the parasite side, excellent *in vitro* model systems are available for the study of differentiation. For the sand fly stages, *in vitro* cultured log phase promastigotes closely resemble procyclic promastigotes, and upon entry into stationary phase they undergo differentiation into a form that closely resembles metacyclic promastigotes (Sacks and Perkins, 1984, Sacks, 1989). For amastigotes, researchers have been able to develop conditions that enable the propagation of amastigote-like forms outside of macrophages, with relative ease and in large quantities (Doyle et al., 1991, Pan et al., 1993, Sereno et al., 2001). While perhaps not identical to authentic lesion amastigotes, most properties are faithfully reproduced, thereby making these a powerful tool for studies of amastigote biology and gene expression.

What is a *Leishmania* virulence gene? In most pathogens, investigators typically define these as ones important for survival and/or pathogenesis of the parasite within the sand fly or mammalian hosts, but not for growth in routine culture media. This definition is not ironclad and exceptions are known (one obvious example arises from the fact that there are dozens of 'routine' culture media). It also allows for the possibility that virulence genes are not 'absolute': that is, it includes genes whose loss shows a quantitative but not complete loss of virulence. In evolutionary terms, even relatively small effects in 'fitness' or virulence can have large

consequences. Moreover, in the natural infectious cycle parasites often experience population bottlenecks, where even fractional decreases in virulence can have profound consequences. For example, sand flies usually transmit no more than 10-100 parasites (Warburg and Schlein, 1986).

The definition of virulence above is an excellent match for experimental studies of *Leishmania*, since the ability of the parasite to grow extracellularly *in vitro* makes it possible to engineer mutations in 'virulence', for subsequent assay(s) in the relevant host(s) and specifically the intracellular amastigotes. Obviously, this definition of virulence genes excludes 'essential' genes, defined as ones required for growth *in vitro*. However, 'essential' genes frequently can play roles in virulence beyond just enabling growth *in vitro*. Given the deadly nature of some *Leishmania* infections, and the desire of investigators to control parasite infections through chemotherapy or vaccination, both 'virulence' and 'essential' genes have great potential in efforts oriented towards the identification of new chemotherapeutic targets.

2. 'Forward' vs. 'reverse' genetic approaches.

Genetic approaches are often divided into two types: 'forward' methods, where one begins with a mutant or variant phenotype and uses this to identify the gene(s) involved, or 'reverse' genetic approaches, where one begins with a parasite gene which is then manipulated in various ways to probe its role in the infectious cycle. In *Leishmania*, both have their advantages and are supported by the requisite genetic tools; the choice is often dictated by factors such as the availability of mutants, the inability to do sexual crosses, and insights or 'intuitions' about genes arising from various sources, etc. One advantage of 'forward' genetics is that it is based solely on the phenotype, making it likely that the genes obtained will be directly involved in the process under study, often with unanticipated roles or functions which would not have been detected by motif searches or database comparisons. While reverse genetic approaches lack this advantage, engineering null mutants is quite rapid and straightforward and thus candidate genes can rapidly

be tested. The development of rapid methods for carrying out reverse genetic approaches across the genome gives them broad potential for screening for new virulence genes. Lastly, even with genes with homologs of known function in other organisms, the ability of these pathogens to utilize standard proteins/motifs in new and unique ways often allows such studies to be rise above simple validation of previously known functions.

A major hurdle for forward genetic approaches in *Leishmania* is that experimentally, these organisms are asexual diploids (Panton et al., 1991). Thus to generate loss of function mutations two genetic events are required, either two independent mutations or a mutation followed by a loss of heterozygosity event (Gueiros-Filho and Beverley, 1996, Hwang and Ullman, 1997). The frequency of these events has been measured in *Leishmania* as ~10^{-6} and 10^{-5} respectively, making the recovery of mutants defective in both alleles relatively rare and on the order of less than 10^{-6} even after mutagenesis (Gueiros-Filho and Beverley, 1996). Thus, to identify mutants powerful screens or selections are required. This is especially a problem if one would like to study mutations affecting 'virulence', as the inability of 'avirulent' mutants to survive makes their recovery difficult for forward genetic selections at present (although the reverse selection towards virulence is quite powerful, as discussed below).

Sexual crossing has been used in many creatures to generate homozygotes through clever mating strategies, and genetic exchange in nature or the laboratory is extremely useful for positional cloning and classical genetic mapping. As mentioned above, experimental crosses amongst *Leishmania* strains or mutants have been unsuccessful (Panton et al., 1991), and there is uncertainty about the extent of genetic exchange in nature. Hybrid parasites have been observed in the field between strains or species (Cupolillo et al., 1997, Belli et al., 1994, Kelly et al., 1991), however whether these represent *bona fide* sexual processes or evolutionarily 'sterile' fusions is as yet unresolved (Torrico et al., 1999). Moreover, population genetic data suggest

that in evolutionary terms, productive genetic exchange is relatively rare (Banuls et al., 1999, Gibson and Stevens, 1999). Experimentally, low or non-existent levels of genetic exchange will prevent recombination from having the opportunity to dissociate changes arising through evolutionary mutations from those associated with the phenotype of interest. Clearly this will hinder and possibly eliminate the use of positional cloning strategies.

Were 'haploid' *Leishmania* available, or methods for systematically making large regions of the genome homozygous in some manner following mutagenesis, our ability to carry out many forward genetic screens or selections would increase greatly. Chromosome fragmentation approaches have been developed recently which yield parasites bearing small hemizygous regions (Tamar et al., 2000, Tamar and Papadopoulou, 2001), and this approach could be used to facilitate forward genetic analysis of these segments.

Alternatively, rather than focus on loss of function mutations one might concentrate on mutations that manifest when heterozygous, either by 'gain of function' or due to haplo-insufficiency. However, the occurrence of either of these following random mutagenesis and for a given gene/phenotype is difficult to predict. A special case of dominant mutations involves the creation of gene fusions to convenient selectable markers or reporter genes, as discussed below by the creation of gene fusion libraries using transposon mutagenesis.

3. Examples of 'forward' genetic approaches to *Leishmania* virulence.

3A. Lipophosphoglycan (LPG) and surface glycoconjugates.

The surface of *Leishmania* promastigotes is coated with a dense glycocalyx, a major portion of which is comprised of the complex glycolipid LPG (Turco and Descoteaux, 1992, McConville and Ferguson, 1993). The structure of LPG is highly conserved amongst *Leishmania* species, showing polymorphism in the composition of branching sugars attached to

the disaccharide phosphate or 'phosphoglycan' repeating units which comprise the bulk of the molecule. LPG is anchored to the parasite surface by a hydrophobic glycosylphosphatidylinositol (GPI) anchor and its outer end terminates with a capping sugar, typically a galactose residue that is capable of binding to appropriate lectins such as ricin agglutinin. Since LPG is effectively the only parasite molecule terminating in galactose, Turco and colleagues used a negative section protocol with ricin agglutinin to isolate a variety of *L. donovani* mutants deficient in LPG biosynthesis (McNeely et al., 1990, McNeely and Turco, 1990). Similar approaches have been taken with other lectins or monoclonal antibodies targeting LPG, in both *L. donovani* and *L. major* (Descoteaux et al., 1998, Cappai et al., 1994, Elhay et al., 1993). At this point in time, more than 12 phenotypically distinguishable LPG mutants of *L. donovani* have been identified (R. Zufferey, S.J. Turco and S.M. Beverley, in preparation; (Beverley and Turco, 1998)).

It should be noted that the lectin-based selection was ideally suited to the challenges posed by 'forward' *Leishmania* genetics, since it enables one to screen very large populations of mutated parasites, as mandated by the relatively low frequency of mutants. It potentially is applicable to any parasite surface molecule for which probes of suitable discrimination and specificity are available, such as GPI anchored proteins.

The LPG-deficient mutants were the starting point for the development of methods and approaches enabling the first functional genetic rescue experiments to be performed in protozoan parasites (Ryan et al., 1993a, Ryan et al., 1993b). Initially, the R2D2 mutant was transfected with a wild-type *L. donovani* library, created in the cosmid shuttle vector cLHYG, and from this population a number of LPG+ transfectants were identified. Analysis of the cosmids present within these led to the identification of the LPG1 gene, encoding a putative Galf transferase responsible for synthesis of the LPG core. Subsequent studies have now extended this approach

to the identification of more than 10 genes involved in LPG biosynthesis, including genes implicated in the synthesis of the polymorphic LPG side chains.

The LPG mutants and genes have provided a resource for probing a number of questions in *Leishmania* biology, including dissection of the biosynthetic pathway of LPG and related glycoconjugates, and testing the role(s) of LPG in parasite virulence. These studies are summarized in two recent reviews (Beverley and Turco, 1998, Turco et al., 2001).

3B. The genetic rescue of avirulence induced by long-term culture *in vitro*.

A powerful approach in studies of prokaryotic virulence has been the identification of avirulent mutants by mass systematic screening of randomly mutagenized populations, and identification of the relevant gene by functional rescue. Unfortunately, the low frequency of mutant recovery in diploid *Leishmania* makes the recovery of such mutants considerably more difficult. To achieve the same level of success achieved in prokaryotic pathogens by screening through thousands of mutants, *Leishmania* workers would have to screen through millions. As yet, high throughput screens for avirulent mutations in sand flies, macrophages and/or mice have not been developed which render this feasible. Their development would clearly constitute a major advance, given the success now achievable with functional rescue methods.

While mutagenesis screens for avirulence are daunting tasks, there is one simple route for creating avirulent lines. Like many pathogens, *Leishmania* has a tendency to lose virulence during *in vitro* culture, at a rate that can be quite rapid in species such as *L. donovani* (unpublished data). Even in *L. major* where virulence is relatively stable, many random clones that were derived from fully virulent lines by a single round of plating showed detectable losses of virulence in mouse infections (Cruz et al., 1993). Neither the genes nor the genetic mechanisms involved in the rapid loss of virulence have been identified, although changes in

abundant surface molecules such as LPG and gp63 have been variably noted by many workers (Greenblatt et al., 1985, Katakura and Kobayashi, 1988, Murray et al., 1990).

In contrast to the difficulty of selecting *Leishmania* for loss of virulence, selections for reacquisition of virulence are readily performed, as the desired parasites are now able to survive and propagate in sand flies, macrophages or mice as appropriate. In unpublished work, our laboratory has been able to partially rescue the defect in mouse infectivity present in an avirulent clonal derivative of the Friedlin V1 strain of *L. major* by genetic complementation (L. Garrity, Y. Wang and S.M. Beverley, in preparation). A number of loci were recovered and their mechanism(s) of action established; none affected LPG or gp63 levels. Our data suggest that the functional rescue was mediated by multi-copy suppression, rather than complementation of a defective A1 strain gene. Whether these loci are able to restore virulence in other culture-attenuated *Leishmania* strains is unknown. These data suggest that when available, attenuated *Leishmania* strains can be productive targets for functional genetic rescue approaches.

3C. Drug resistance and its application to virulence.

Selection for drug-resistant *Leishmania* has been widely used to develop parasite mutants showing a variety of genetic modifications including gene amplification and various kinds of gene mutations and/or inactivations (Beverley, 1991, Ouellette et al., 1996). Gene amplification appears to be especially common in *Leishmania*, whereas it is rare in trypanosomes. This may reflect differences in the requirement of *Leishmania* for the use of RNA polymerase II promoters or replication origins on episomal amplified DNAs.

In most instances, investigators have been able to track down the gene (or genes) responsible for drug resistance. Examples included amplified genes such as the bifunctional dihydrofolate reductase-thymidylate synthase, pteridine reductase, P-glycoproteins, ornithine decarboxylase, and IMP dehydrogenase (Hanson et al., 1992, Wilson et al., 1991, Beverley et al.,

1986, Beverley et al., 1984, Callahan and Beverley, 1991, Papadopoulou et al., 1992, Ouellette and Borst, 1991, Bello et al., 1994). This has also been possible in the case of point mutations leading to loss (Vasudevan et al., 1998) or gain of function (Arrebola et al., 1994). Recently, selections of *Leishmania* transfectant libraries bearing multicopy episomal cosmids (each containing ~40 kb sections of the ~34 Mb genome) have been employed to identify genes able to mediate resistance when overexpressed or amplified (Cotrim et al., 1999, Kundig et al., 1999). One advantage of this approach is that in theory each cosmid-borne gene is effectively 'pre-amplified', making their occurrence approximately 10^{-3} in the transfectant library. In contrast, the frequency of gene amplification is much less ($<10^{-6}$) and typically only occurs after several rounds of stepwise selection (Borst and Ouellette, 1995, Beverley, 1991). The cosmid library transfectant selection approach has been applied to the recovery of genes mediating resistance to antifolates, toxic nucleosides, and drugs inhibiting sterol biosynthesis (Cotrim et al., 1999, Kundig et al., 1999).

While drug resistance is obviously a useful tool for exploring metabolic pathways and potential parasite responses to current or prospective chemotherapy, is it relevant to the study of virulence *per se*? First, as is now clear for many pathogens, many metabolic pathways in *Leishmania* are often intimately involved in parasite virulence, beyond their known roles in viability. While the trypanothione reductase gene is essential, heterozygotes containing only a single copy grow normally *in vitro* but are attenuated in macrophage infections (Dumas et al., 1997). As will become clear below, surprises can occur. One example is the pteridine reductase (PTR1) gene, which is essential *in vitro* in defined media (Bello et al., 1994), but *in vivo* appears to be dispensable, presumably because the mammalian host synthesizes reduced pteridines *de novo* (Cunningham et al., 2001). Remarkably, loss of PTR1 expression gave rise to increased pathogenesis in mouse infections, through an elevation of the rate at which parasites differentiate

to the infective metacyclic form (Cunningham et al., 2001). Application of inhibitors of other pathways potentially involved in virulence may prove equally revealing.

A second reason is that aspects of the complex interplay amongst host and parasite can often be replicated (at least in part) by drug selections *in vitro*. *Leishmania* typically faces powerful chemical stresses following macrophage invasion, involving both reactive oxygen and nitrogen intermediates. These defenses can be replicated conveniently *in vitro* by agents such as hydrogen peroxide, primaquine, paraquat, xanthine/xanthine oxidase, and a variety of NO donors. We have used selections with these agents of cosmid transfectant libraries to identify several loci implicated in resistance to one or more of these agents (L.A. Garraway, B. Nare and S.M. Beverley, unpublished data; (Ellenberger and Beverley, 1989, Bello et al., 1994)). One could easily extend this concept to other 'chemical' defenses of the macrophage, such as low pH, toxic peptides (defensins), and proteases, all of which could be employed in selections *in vitro*. Lastly, during invasion, parasites undergo complex signaling responses that initiate and coordinate their defense mechanisms (Reiner, 1994), often involving protein kinase cascades. Many of these parasite 'signaling' enzymes are known to be susceptible to inhibitors previously developed in other systems, and thus potentially amenable to identification through a drug selection approach.

3D. Forward genetics by transposon mutagenesis.

Transposon mutagenesis is an extremely powerful form of 'forward genetics', where mutations are generated by the introduction of transposable elements (Berg, 1989). Amongst its 'forward' genetic applications, transposon mutagenesis is often used for insertional inactivation, where the transposon itself can marking the relevant gene for subsequent cloning. Unfortunately, this powerful approach is poorly suited for applications in asexual diploid organisms such as *Leishmania*: since two events are required to inactivate a gene, the level of

transposition needed to attain this at a given locus is such that the genome becomes quickly littered with irrelevant heterozygous transposition events.

A second common application of transposon mutagenesis is for the creation of gene fusions, for example to convenient selectable markers or reporter genes whose expression can be rapidly manipulated. While gene fusions can be generated by a variety of routes *in vitro* and *in vivo*, the extremely low frequency of nonhomologous recombination in *Leishmania* and other trypanosomatids (Cruz et al., 1991, Cruz and Beverley, 1990, Clayton, 1999, Swindle and Tait, 1996) precludes the use of simple DNA transfection-based approaches *in vivo*. Transposition conveniently overcomes this limitation.

In *Leishmania*, expression of the *Drosophila mariner* transposase leads to mobilization of *mariner* elements, which can be engineered to yield relevant gene fusions that can be selected or screened for *in vivo* (Gueiros-Filho and Beverley, 1997). The *mariner* transposase has been purified and shown to catalyze transposition *in vitro*, allowing the creation of transposon insertion libraries in cosmid shuttle vectors for subsequent analysis following transfection into *Leishmania* (Tosi and Beverley, 2000). Recently we have created a variety of useful modified *mariner* elements, bearing a variety of selectable markers and reporter genes that are suited for the selection of both transcriptional and translational gene fusions (Goyard et al., 2001). *In vitro* transposition systems based upon other mobile elements such as bacterial Tn5 or yeast Ty1 have been developed, and could be similarly applied.

By one route or another, libraries of genes fused to a convenient reporter protein (such as β-galactosidase or GFP) are generated, and then scored for expression. In prokaryotes the GFP-based approach is termed differential fluorescence induction (Valdivia et al., 1996). Notably, while most gene fusion libraries emphasize regulation at the mRNA or 'transcriptional' level, one can design fusions that only work at the protein or 'translational' level. Such fusions are

ideal for systematically studying expression broadly across the entire 'proteome'. Since bacteriologists began their elegant studies prior to the current 'omics' era, one could consider the application of 'translational' gene fusions to the study of protein expression as 'proteogenomics' (Beverley et al., 2001). While characterization of gene fusions is not strictly a study of 'virulence' genes, a common expectation is that genes or proteins showing changes in expression as cells move through their infectious cycle are likely to play significant roles in that process.

4. Reverse genetics and *Leishmania* virulence.

By definition, reverse genetics starts with genes and works back towards mutants and hopefully, phenotypes. There are many criteria for picking genes to pursue: developmental regulation, abundance of encoded proteins, provocative sequence 'motifs' or phylogenetic relationships, cellular localization, or enzymatic activity have all been productively applied. Today, the fields of genomics and proteomics (and possibly other '–omics') are now providing an abundance of genes and/or proteins, many of whose functions are completely unknown and thus ripe for study.

4A. The *Leishmania* genome project.

The *Leishmania* genome comprises about 34 megabases and is estimated to encode about 10,000 genes (Blackwell and Melville, 1999, Myler and Stuart, 2000). An international consortium of researchers is determining the genome sequence of a prototypic species, *Leishmania major*, and completion is anticipated in a few years time (Myler and Stuart, 2000, Ivens et al., 1998). As of August 2001, more than 5 Mb of finished and 25 Mb of unfinished sequence is available, allowing researchers to scan for genes of interest by a variety of approaches.

4B. Tools for manipulating the *Leishmania* genome.

Procedures required for the application of reverse genetic approaches are well developed in *Leishmania*. These include a variety of selectable markers, reporter genes, and expression vectors, homologous gene replacement, inducible expression systems, transposon mutagenesis, artificial chromosomes and chromosome fragmentation approaches. These are summarized in Table I and the reader is referred to the references cited for more information. Investigators can now readily probe gene function through effects arising from overexpression, mutation, and deletion.

One challenge remaining is the study of 'essential genes', whose loss by definition cannot be tolerated by the organism. The difficulty here is discerning when a gene is 'essential' and thus cannot be eliminated, from simple technical difficulties. Remarkably, attempts at knocking out 'essential' genes have yielded the planned replacements, but accompanied by expansion of the target gene number through changes in chromosomal number or ploidy (Cruz et al., 1991). Since this is a rare event normally, the recovery of planned replacements retaining a wild-type gene through chromosome number increase has been used as a positive criterion for gene essentiality (Mottram et al., 1996b, Dumas et al., 1997). Another possibility is the use of regulatable systems, where genes are placed under the control of elements imported from other species such as the *E. coli* tetracycline or *lac* operon repressors (Yan et al., 2001). These will allow investigators the more powerful option of creating conditional mutants for the study of essential genes. Since *Leishmania* virulence assays in mice typically take months, whether the available regulatory systems will be up to usage over this extended time frame remains to be established. Fortunately and as noted earlier, many genes required for parasite virulence in flies or mammals are not essential in the more forgiving environment of the culture flask *in vitro*.

One approach that has proven extremely powerful in metazoans and trypanosomes has made use of the phenomenon of RNA interference (RNAi), where introduction or expression of short double-stranded RNAs leads to the rapid destruction of cognate mRNAs (Ngo et al., 1998). This approach has a number of advantages: it is fast, requires very little sequence information, and it is able to reduce expression from multiple gene copies (which is especially advantageous in asexual diploids) (Wang et al., 2000, Shi et al., 2000, LaCount et al., 2000, Ngo et al., 1998). Thus far success with RNAi has not been reported in *Leishmania*, and there is one report showing that an RNAi-mechanism was not involved in successful antisense inhibition of gene expression (Zhang and Matlashewski, 2000).

4C. Examples of "reverse genetic" tests of candidate *Leishmania* virulence genes.

At this point in time the number of *Leishmania* genes that have been subjected to reverse genetic analysis is large and growing rapidly. A number of examples relevant to the study of virulence are included in Table 2, illustrating the power of this approach. Many of the studies yielded the expected phenotype: the amastigote-specific *L. donovani* A2 gene was important for macrophage survival, as were *L. major HSP100* and *L. mexicana* cysteine proteinases (Zhang and Matlashewski, 1997, Alexander et al., 1998, Hubel et al., 1997). Remarkably, there have been a number of surprises. For example, *Leishmania* gp63 is the most abundant promastigote surface protein and is encoded by a large gene family (Medina-Acosta et al., 1993). However, targeted deletion of the gp63 gene cluster of *Leishmania major* yielded only minor phenotypic effects *in vitro* (affecting deposition of complement) but no effects in sand fly, macrophage or mouse infections (Joshi et al., 1998). Similarly, deletion of the cluster encoding the *SHERP/HASP* genes, which encode abundant metacyclic proteins in *L. major*, had little effect (McKean et al., 2001), as did loss of all GPI anchored proteins in *L. mexicana* (Hilley et al., 2000).

One of the most remarkable findings is that putative virulence genes/molecules are not equally active in all *Leishmania* species. In *L. major*, deletion of *LPG1* (encoding a putative galactofuranosyltransferase required for the synthesis of the heptasaccharide core of LPG) yielded promastigotes that were specifically deficient in LPG biosynthesis (Spath et al., 2000). These parasites were unable to survive in sand flies or efficiently establish infections in mice or macrophages (Spath et al., 2000, Sacks et al., 2000). Conversely, in *L. mexicana* LPG1 deletions had little if any phenotype in mouse or macropahge infections (Ilg, 2000). A similar discrepancy was reported between these two species with null mutants of *LPG2*, which are unable to synthesize all phosphoglycan repeating units due to loss of the Golgi GDP-mannose transporter (Ilg et al., 2001, Turco et al., 2001). It is safe to state that most investigators expected the phenotype of LPG pathway mutations to be similar in all *Leishmania*. A similar contrast has been seen with gp63: some attenuation was observed in *L. amazonensis* but not *L. major* (Chen et al., 2000). While it is tempting to speculate that the differences observed reflect experimental shortcomings, these have been carefully considered and seem unlikely at present. Perhaps equally informative is the opinion of the host: just as for the parasite, mutations affecting the host immune response can have dramatically different impacts on parasite virulence in *L. major* and *L. amazonensis* (Turco et al., 2001).

These findings make the point that far from being monotypic, different *Leishmania* species make use of their repertoire of potential 'virulence' genes and molecules to different extents in their interactions with the host. Why this should be the case, and whether this reflects the use of convenient animal model systems, rather than the natural hosts (where the pathology can differ considerably from that of the laboratory mouse), remains to be determined. Given that these parasites differ considerably in many aspects of their biology and disease pathology, perhaps these findings should not have been a surprise.

5. 'Reverse genetics at warp speed': Functional genomics, computational biology and bioinformatics.

The coming availability of the entire complement of ~10,000 *Leishmania* genes poses a considerable challenge to traditional gene-by-gene reverse genetic approaches. The emerging field of 'functional genomics' is devoted to the development and application of methods which allow investigators to efficiently study many genes simultaneously. For example, changes in gene expression can be monitored through the application of DNA microarray technology across thousands of genes (Schena et al., 1995, Brown and Botstein, 1999). Genomic resources suitable for DNA microarray approaches in *Leishmania* are now available and being used to study developmental gene expression (Akopyants et al., 2001, Beverley et al., 2001). Similarly, proteomic methods that allow investigators to view *Leishmania* protein expression are now beginning to be employed. Lastly, as more genes are characterized functionally in *Leishmania* and other organisms, increasingly investigators are able to bring to bear powerful computational approaches to identify candidate genes for more intensive analysis.

Functional genomics, proteomics and computational biology offer two promises for the future: the more obvious one perhaps is a way of prioritizing which genes should be the focus of more intensive studies. As these fields advance however, they promise to bring new directions that will be both distinct and complementary to the gene-by-gene' approach.

6. Validation of candidate virulence genes: requirements and challenges.

Once the investigator has identified a candidate virulence gene, and modified its expression by one of the methods described above, appropriate tests to confirm its role in virulence are necessary. Stanley Falkow proposed a set of "Molecular Koch's Posulates" that provide an excellent standard suitable for use in molecular genetic studies of *Leishmania* virulence (Falkow, 1988). First, the candidate gene must be reasonably involved in processes

thought to be essential to virulence; second, inactivation of the gene should lead to a loss of virulence, and third, restoration of gene function should lead to restoration of virulence. The first two are perhaps obvious to most investigators, however the importance of the last one cannot be underestimated. Like many pathogens, *Leishmania* is known to lose virulence spontaneously during culture *in vitro*, and many manipulations of the parasite are themselves mutagenic (including DNA transfection). Thus parasites may lose virulence through processes unrelated to those planned by the investigator, making fulfillment of the last criterion essential in establishing the role of a putative virulence gene. Many but not all of the studies summarized in Table 2 have employed these criteria.

Remarkably, restoration of gene function can be more challenging than one might expect, despite the availability of an extensive repertoire of *Leishmania* expression vectors (Table 1). Most of the available vectors lead to overexpression of the inserted gene, which in some cases is as detrimental as underexpression. This has been observed in several cases in *Leishmania* (Hubel et al., 1997, McKean et al., 2001). Thus care must be taken in applying the last criterion to ensure that the expression of the restored gene is physiologically relevant. In some cases, it may be necessary to 'reintegrate' the gene back into the target locus in order to ensure proper temporal and quantitative expression.

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Table 1. A summary of genetic tools available for use in *Leishmania*

	Examples	References
Reporter genes	*LACZ, GUS, CAT, GFP*	
Transient transfection	CAT, LACZ, GUS	(Laban and Wirth, 1989, LeBowitz et al., 1991)
Stable transfection & markers		
Positive selection	*NEO, HYG, PHLEO, PAC, SAT, BSD, NAGT, DHFR-TS*	(Cruz and Beverley, 1990, Cruz et al., 1991, Freedman and Beverley, 1993, Joshi et al., 1995, Brooks et al., 2000, Goyard and Beverley, 2000, Liu and Chang, 1992, Arrebola et al., 1994)
Negative selection	*TK, CD*	(LeBowitz et al., 1992, Muyombwe et al., 1997)unpublished data)
Expression vectors		
Circular episomes	pX series	(LeBowitz et al., 1990, Ha et al., 1996)
Chromosomally integrated	pIR1SAT, pSSU-int	(Misslitz et al., 2000); unpublished data)
Regulatable (inducible)	*tet* repressor system	(Yan et al., 2001)
Homologous gene replacement		
Single copy genes	*DHFR-TS*	(Cruz and Beverley, 1990)
Gene arrays	*GP63, CYPB, aTUB*	(de Lafaille and Wirth, 1992, Joshi et al., 1998, Mottram et al., 1996a)
Transposon mutagenesis		
in vivo	*mariner*	(Gueiros-Filho and Beverley, 1997)
in vitro	*mariner*, Ty1	(Tosi and Beverley, 2000, Garraway et al., 1997)
Loss of heterozygosity (LOH)	*DHFR-TS, HGPRT, APRT*	(Gueiros-Filho and Beverley, 1996, Hwang et al., 1996)
Chromosome fragmentation	*TR, PTR1*	(Tamar and Papadopoulou, 2001)
Functional genetic rescue	LPG biosynthetic genes	(Beverley and Turco, 1998, Turco et al., 2001)
Artifical chromosomes (LACs)	*DHFR-TS* based LAC	Unpublished data
RNA interference (RNAi)	Not in *Leishmania* to date (many successes in trypanosomes)	(Zhang and Matlashewski, 2000)

Table 2. Examples of reverse genetic studies of candidate *Leishmania* virulence genes.

Gene	Species	Approach	Attenuation in fly/mouse/MF infections	Reference
GP63	*L. major* *L. amazonensis*	KO antisense	Little if any ++	(Joshi et al., 1998) (Chen et al., 2000)
A2	*L. donovani*	antisense	++++	(Zhang and Matlashewski, 1997)
Mannose biosynthesis *PMI* (phosphomannoisomerase) *GDPMP* (GDP-mannose pyrophosphorylase)	 *L. mexicana* *L. mexicana*	 KO KO	 ++ ++++	 (Garami and Ilg, 2001b) (Garami and Ilg, 2001a)
Cysteine proteinases (*CPA+CPB*)	*L. mexicana*	KO	++++	(Alexander et al., 1998)
Protein GPI anchors (*GPI8*)	*L. mexicana*	KO	Little if any	(Hilley et al., 2000)
LPG biosynthesis *LPG1* *LPG2*	 *L. major* *L. mexicana* *L. major* *L. mexicana*	 KO KO KO KO	 +++ Little if any ++++ Little if any	 (Spath et al., 2000, Sacks et al., 2000) (Ilg, 2000) (Späth, Turco, Sacks & Beverley, unpublished) (Ilg et al., 2001)
SHERP/HASP	*L. major*	KO Overexpression	Little if any +++	(McKean et al., 2001) (McKean et al., 2001)
HSP100	*L. major*	KO	++	(Hubel et al., 1997)
TR (trypanothione reductase)	*L. donovani* *L. major*	Heterozygote (+/deletion)	++	(Dumas et al., 1997)

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