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**Cornelia Hoyer · Katja Mellenthin · Markus Schilhabel
Mathias Platzer · Joachim Clos**

Use of genetic complementation to identify gene(s) which specify species-specific organ tropism of *Leishmania*

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Abstract We have employed a genetic complementation screening to identify genetic markers of heat stress tolerance and visceralisation of *Leishmania* infection. *Leishmania major*, which has a low thermotolerance and which causes cutaneous lesions, was transfected with a cosmid library of *L. donovani* DNA. The recombinant parasites were then screened either for thermotolerance or selected by repeated passage in BALB/c mice. Cosmids which conferred selective advantage were isolated. Several strategies were tested to identify the gene(s) within the cosmids responsible for the observed selective advantages. Of the approaches tested, the complete sequence analysis of the cosmids and subsequent screening of defined candidate ORFs proved to be the method of choice. Other approaches, such as creation of sub-libraries or transposon insertion strategies proved to be unsuccessful.

Thermotolerance and tropism

There is a consistent correlation between the tropism of *Leishmania* spp. and their temperature tolerance profile. Viscerotropic species can tolerate temperatures of $>37°C$, the permissive range of cutanotropic species is limited to $<35°C$. This difference in temperature tolerance can be observed both for intracellular amastigotes and for promastigotes of the respective species [1, 2]. While this correlation is unquestioned, it remains to be investigated whether temperature tolerance is indeed a determinant of organ tropism. One obvious way to solve this problem is to produce strains of a cutanotropic *Leishmania* species, e.g. *L. major*, with an increased temperature tolerance. Such strains could then be tested for their viscerotropic potential in infected laboratory animals.

There are several ways to address this question. Candidate genes can be selected by their presumed function, e.g. in thermotolerance/heat stress tolerance. The genes could be overexpressed in *L. major* to create strains with increased thermotolerance. Reversely, such genes could be replaced by homologous recombination to test whether gene loss leads to lowered thermotolerance. This approach was followed when we replaced the ClpB genes of *L. major* and *L. donovani*, which encode the 100-kDa heat shock protein (Hsp100). This heat shock protein, by analogy, was predicted to confer inducible stress tolerance, similar to its homologues in yeast and *E. coli*. Nevertheless, loss of the gene did not produce a thermotolerance phenotype in *L. donovani* and only a moderate effect in *L. major* [3, 4]. Overexpression of Hsp100 also did not alter the permissive temperature range of these parasites. It thus became clear that selecting genes for reverse genetic analysis by analogy to known homologues is risky. Moreover, the prejudiced analysis of pre-selected genes may miss the key genes altogether.

We therefore implemented a genetic complementation screen. A cosmid library of *L. donovani* genomic DNA was constructed in the shuttle vector pcosTL [5]. The average insert size is approximately 38 kb and, by hybridisation screening with various DNA probes the library was found to be representative of the *L. donovani* genome. We calculate that, given a standard Poisson-type distribution, 4,360 individual cosmid clones represent the *L. donovani* genome with a 99% confidence. DNA of the cosmid library was purified by standard procedures [6] and used for electrotransfection of *L. major* strain 5ASKH, a strain with robust virulence in previous experiments [3].

In the first line of experiments, recombinant *L. major* promastigotes were exposed to the non-permissive temperature of 37°C for up to 6 days. After this selection,

C. Hoyer · K. Mellenthin · J. Clos (✉)
Bernhard Nocht Institute for Tropical Medicine,
Hamburg, Germany
E-mail: clos@bni.uni-hamburg.de

M. Schilhabel · M. Platzer
Institute for Molecular Biotechnology, Jena, Germany

cultures containing wild-type *L. major* did not show recovery. The recombinant promastigotes, however, gave rise to survivor populations indicating that the cosmid(s) retained in that population conferred a selective advantage. Cosmid DNA was isolated from the survivor populations using a standard plasmid DNA minipreparation protocol and used to transform competent *E. coli* DH5. Colonies were screened by DNA minipreparation and restriction endonuclease analysis to determine the distribution of cosmids within the survivor population. Only one or two cosmid species could be isolated in each screen, which indicates that the selective pressure was specific. In 20 independent temperature-selection screens, ten individual cosmids were identified of which clones were established. Southern analysis showed that not one of these cosmids overlapped with any of the other cosmids or with known heat shock genes of *L. major*. This is indication that temperature tolerance in *Leishmania* is probably a multigenic feature and cannot be attributed to a single gene locus.

Clonally selected cosmids representing each cosmid species were transfected individually into wild-type *L. major*, creating defined transfectant lines. Parasites from each line, plus parasites transfected with the cosmid vector, pcosTL, were mixed at equal numbers and subjected to a secondary competitive selection at 37°C for 4, 5, and 6 days. Survivors were amplified at 25°C and cosmid DNA was isolated. After transformation into *E. coli*, the cosmids of bacterial clones were analysed by restriction endonuclease digestion to determine the distribution of the preselected cosmids after the secondary screening. Figure 1 shows the distribution of cosmids after 4, 5, or 6 days of selection. Obviously, the selective advantage conferred to *L. major* by the various cosmids differs to some degree.

The same mixture of individually transfected *L. major* were also inoculated into the footpad of BALB/c mice. After 10 weeks of infection parasites were isolated from lymph node and spleen tissue and individually grown in liquid culture as promastigotes. Cosmid distribution within the re-isolate populations was tested in analogy to the temperature screens. Figure 2 shows the result of this in vivo selection. Three of the cosmids favoured in the temperature tolerance screens (Fig. 1) also conferred some selective advantage in vivo. There was a marked difference, however, between in vivo and in vitro data for cosmids 11.2 and 8.0. While cosmid 11.2 was dominant after 4 days of temperature selection, it did not confer any advantage during mouse passage. In contrast, cosmid 8.0, which did not confer a marked advantage during in vitro temperature selection, was most favoured in the in vivo screening. This analysis showed that, while cosmids which confer increased thermoresistence may also increase viability inside the murine host, the two traits are not necessarily linked.

This prompted us to develop a second selection strategy. *L. major* promastigotes were transfected with the DNA of the *L. donovani* cosmid library. After G418 selection for stably transfected parasites, the mixed

Fig. 1 Temperature selection of recombinant *Leishmania major*. Ten defined recombinant *L. major* strains and a strain with the parent cosmid vector pcosTL were mixed at equal ratio and exposed to 37°C for 4, 5 and 6 days. The cosmid DNAs from survivors were prepared after proliferation at 25°C and transformed into *E. coli*. The distribution of the different cosmid species was determined by restriction analysis of the DNA of 50 bacterial colonies each

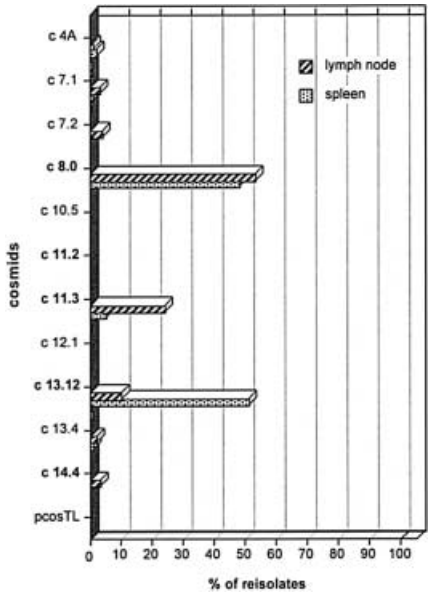

Fig. 2 Selection of recombinant *L. major* by passage in mice. BALB/c mice were infected with a mixture of 12 recombinant *L. major* strains. The strains were transfected with clonally selected cosmids from the temperature tolerance screening. The mixture also included a strain containing a cosmid with the *ClpB* locus (cos 4A) and a strain transfected with the cosmid vector pcosTL. Parasites were re-isolated from spleens and draining lymph nodes 10 weeks after infection. Following proliferation of the parasites in vitro the cosmid DNA was isolated and transformed into *E. coli*. Fifty bacterial clones for each re-isolate were subjected to cosmid DNA minipreparation and restriction analysis

recombinant population was inoculated in the footpad of BALB/c mice. After 7 weeks, parasites were re-isolated from lymph node and spleen. Both re-isolates were re-injected into mice for a second round of selection. Parasites were cultivated from spleen tissue after 7 weeks of infection. The cosmid distribution within the selected populations was analysed and compared. There was a striking dominance of one cosmid species, pcosM0, in the parasites re-isolated in two consecutive rounds from spleen (Fig. 3). In the isolates selected once through lymph node and once through spleen, in contrast, no individual cosmid was dominant, although cosmid pcosM0 was found. The dominant selection for pcosM0 in the spleen of infected mice could be verified in further repeated screenings (not shown). Analysis with clonal isolates of *L. major* (pcosM0) showed that this cosmid does not confer increased virulence in isolated macrophages in vitro. The effect of cosmid pcosM0, therefore, seems to be specific for survival within the visceral organs.

Our strategy of genetic complementation screenings revealed, in a first approximation, that the two traits selected for, thermotolerance and visceralisation potential, can (but not necessarily have to) select for the same genes. This is indication for a more complex genetic basis for these traits.

We tested several strategies to narrow in on the genes responsible for the selective advantage conferred by the cosmids isolated in the selection screens. Secondary selection of plasmid sublibraries constructed from random digests of individual cosmids did not yield parasite strains with increased thermotolerance. We assume that random fragments are not likely to contain both the complete open reading frame (ORF) of a gene as well as the complex and poorly understood regulatory elements that govern its timely expression.

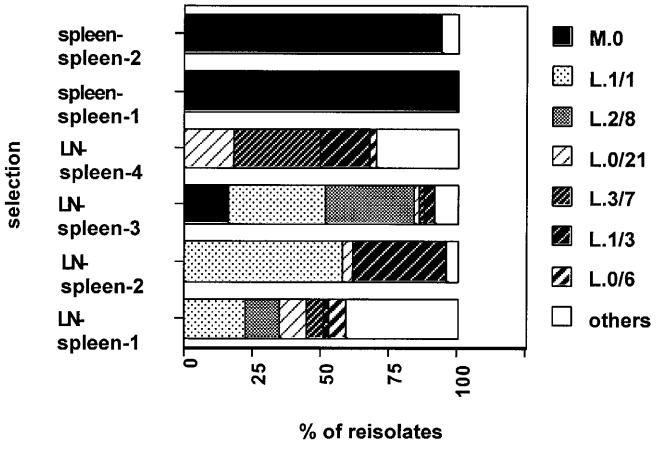

Fig. 3 Selection of recombinant *L. major* by repeated mouse passage. *L. major* promastigotes were transfected with cosmid DNA from an *L. donovani* genomic DNA library. Recombinant parasites were inoculated into BALB/c mice. 7 weeks after infection parasites were re-isolated from lymph node and spleen. Both re-isolates were re-inoculated into BALB/c mice. After 7 weeks, parasites were re-isolated from spleen and amplified in vitro. Cosmid DNA was isolated and used to transform *E. coli*. Fifty bacterial clones per isolate were analysed by restriction digest and gel electrophoresis to determine the distribution of cosmids. Isolates marked with "spleen-spleen" were isolated from spleen both in the first and second round of selection. Isolates marked *LN-spleen* were recovered from lymph node first, and, in the second round of selection, from spleen

We also examined the possibility for random integration of transposons in vitro, both for sequence analysis and for reverse selection. Sequencing results demonstrated, however, that the Tn7 transposon used integrated preferentially in the cosmid vector and not in the G/C-rich *Leishmania* DNA inserts.

We therefore subjected the cosmids to high-throughput sequencing. Contigs were established for each cosmid insert and putative ORFs were identified. We were helped in this regard by the fact that *Leishmania* genes are grouped in large, polycistronic transcription units, which results in an absolute strand bias ([7], see also Myler et al., this issue). Therefore, only ORFs were accepted that were colinear to other ORFs, which in turn showed homology to known genes in the database.

Individual ORFs were fused in the integration vector pIRmcs+ (derivative of pIRSAT, a gift from S. Beverley) and integrated in the rDNA locus of individual *L. major* strains. These strains are currently under selection, both for temperature tolerance and for virulence/tropism in BALB/c mice.

Once the selective advantages can be attributed to defined gene(s), the question can be addressed whether the effects are due to increased gene dosis, specific characteristics of gene alleles found in *L. donovani*, or whether the gene(s) responsible are exclusive to *L. donovani*.

Genetic complementation has been used in *Leishmania* previously to identify the components of the glycoconjugate synthesis pathways [8, 9, 10, 11]. The approach is applicable to all traits that allow a stringent selection, including virulence and drug resistance. We have recently applied the methodology to the selection of genes that mediate resistance to ansamycin antibiotics (Wiesgigl and Clos, submitted). Compared with the selection for spontaneous resistance, the selection of escape mutants from a pool of stable transfectants is faster and can be analysed with less ambiguity. Therefore, complementation genetics is a valuable tool to analyse the molecular basis of genetic traits in pathogenic microorganisms.

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