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Identification of a Genomic Fragment of *Mycobacterium tuberculosis* Responsible for In Vivo Growth Advantage

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Although *Mycobacterium tuberculosis* is one of the oldest known human pathogens, little is known about mycobacterial pathogenesis. The ability to transfer and express genes among the mycobacteria, first documented in 1987 (1) allows the application of genetics to the elucidation of this microbe's pathogenic determinants. We sought to develop a system based on genetic complementation in vivo, using integrating shuttle cosmid libraries, to identify potential virulence genes of *M. tuberculosis*. An existing pair of *M. tuberculosis* strains, H37Rv (virulent) and H37Ra (avirulent), distinguishable by their ability to cause disease in animal models (6), were used.

Virulence is a complex phenotype resulting from the expression of many genes that are often coordinately regulated in response to different environmental signals (4). Hence, a strategy to identify virulence determinants by genetic complementation requires two strains that are genetically similar and a phenotype associated with virulence. H37Ra and H37Rv were derived from the same clinical isolate in 1934, (8,9). and their macroscopic genome organization is similar (M. Tuckman et al., unpublished observation). Based on described differences in growth rates of H37Ra and H37Rv in mouse lung and spleen and evidence that established a correlation of growth rate and pathogenicity of *M. tuberculosis* (5,7), we defined the ability of recombinants to grow faster than H37Ra in the mouse as a potential virulence phenotype.

Our strategy was to infect mice with pools of H37Ra recombinants containing H37Rv DNA in integrating cosmids to allow the selection of growing clones in mouse spleen and lung. The integrating shuttle cosmid libraries were ideal for in vivo complementation because, theoretically, only 225 clones are required to represent the H37Rv genome, toxic effects associated with the expression of genes from multicopy plasmids are avoided, kanamycin selection pressure is not necessary to maintain the cosmid, and many contiguous genes may be expressed.

To confirm that the in vivo selection was not random, the growth rates of selected clones were measured in mouse spleen and lung, and a method was developed to retrieve the H37Rv insert DNA from the chromosome of a recombinant.

MATERIALS AND METHODS

Construction of H37Ra/H37Rv Recombinant Pools

A genomic library of H37Rv was constructed in the integrating shuttle cosmid vector pYUB178 that contains an *E. coli ori;* the L5 mycobacteriophage attachment site *att*P; the L5 integrase gene *int;* a kanamycin resistance gene, *aph*, derived from Tn903, lambda *cos*, and a unique cloning site, *Bcl* I.

An 8-day-old H37Ra culture was electroporated with pYUB178::H37Rv DNA, library DNA, and, separately, pYUB178 DNA. Two pools of H37Ra-(pYUB178::H37Rv) recombinants were made by collecting and inoculating ~225 colonies into 50 ml of enriched Middlebrook 7H9 broth containing 10 μg/ml kanamycin. Growth occurred at 37°C for ~2 weeks. At the same time, the H37Ra(pYUB178) control was prepared as a pool of 14 transformant colonies. Samples of pools were distributed and fro-

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TABLE 1. Mycobacterium tuberculose *strains and plasmids*

M. tuberculosis strain, clone, or plasmid	Description
H37Ra	Avirulent strain
H37Rv	Virulent strain
mc²816	H37Ra containing pYUB178
mc²806	H37Ra containing pYUB178::H37Rv *ivg*1
mc²807	H37Ra containing pYUB178::H37Rv *ivg*2
mc²822	H37Ra containing pYUB353
mc²823	H37Ra containing pYUB354
mc²824	H37Ra containing pYUB355
mc²825	H37Ra containing pYUB356
pYUB178	Integrating shuttle cosmid vector
pYUB352	H37Rv *ivg*1-containing cosmid derived from mc²806
pYUB353	pYUB178::H37Rv *ivg*1
pYUB354	pYUB178::H37Rv *ivg*1
pYUB355	pYUB178::H37Rv
pYUB356	pYUB178::H37Rv

zen in cryovials for later use. See Table 1 for the description of *M. tuberculosis* strains, clones, and plasmids used in this study.

Mouse Infection

Groups of 55 C57BL/6 mice, age 6–8 weeks, were intravenously inoculated with each recombinant group or control group per time point. Inoculation of mice with spleen-passaged bacteria was accomplished by first homogenizing infected spleens after 14 days in 5 ml sterile saline. One milliliter of the 5-ml spleen homogenates from each of the five mice per group was pooled and filtered through sterile gauze to exclude tissue clumps. The filtrate was used to directly inoculate another set of mice.

RESULTS AND DISCUSSION

Selection of Growing Recombinants from Pools

Individual recombinant clones were picked from plated lung homogenates of mice infected with spleen-enriched pools. Of the 450 recombinants that were passed through mice, only seven clones were detected in lung after spleen enrichment (data not shown). This in vivo selection could be explained by one of two possibilities: (a) the in vivo–selected clones replicated in mouse lung and spleen, and unselected clones did not or (b) the in vivo–selected clones persisted longer than unselected clones in spleen and lung. To distinguish between the possibilities, in vivo growth measurements of two of the clones, mc²806 and mc²807, were compared with those of H37Ra containing pYUB178 (mc²816) and H37Rv (Table 2).

These data show that in vivo passage of recombinant pools enriched for individual clones that specifically grew during the first 2–4 weeks of infection. The in vivo strategy employed in this study yielded clones with two phenotypes. Clone mc²806 has a growth advantage in the spleen, and mc²807 has a growth advantage in the lung and spleen. The fact that the growth rates of the clones were not equal to that of H37Rv suggests that more than one H37Rv locus was most likely required to restore full virulence to H37Ra.

Retrieval of a H37Rv DNA Insert That Confers In Vivo Growth Advantage to H37Ra

To prove that the H37Rv DNA insert present in an in vivo selected recombinant was responsible for its in vivo growth advantage, it had to be retrieved from the chromosome. Because the pYUB178::H37Rv are integrated into the chromosomes of H37Ra recombinants, the H37Rv insert DNA cannot be easily removed. A method was devised to clone the H37Rv DNA insert as a cosmid. The retrieval procedure is very efficient; 32 of the 33 mc²806-retrieved cosmids contained the entire H37Rv insert (data not shown). The cosmid clone designated pYUB352 was used for further study.

Identification of pYUB352-Overlapping Cosmids from the pYUB178::H37Rv DNA Library

The next step was to reintroduce retrieved cosmids into H37Ra to measure the growth rates of

TABLE 2. *In vitro growth of* M. tuberculosis *recombinants*

Experiment	Clones tested	Growth during first 2 weeks of infection
J33	mc²806	Spleen growth
		Lung growth
	mc²807	Spleen growth
		Lung growth
	mc²816	No growth in spleen
		No growth in lung
	H37Rv	Spleen growth
		Lung growth
J36	mc²806	Spleen growth
	mc²822	Spleen growth
	mc²823	Spleen growth
	mc²824	No growth in spleen
	mc²825	No growth in spleen
	mc²816	No growth in spleen
	H37Rv	Spleen growth

recombinants. Retrieved cosmids did not contain the *int* gene; it was lost during the cloning procedure. To identify integrating cosmids that shared the H37Rv DNA insert of mc^{2}806, pYUB352 was used as a probe to screen the pYUB178::H37Rv library in *E. coli* for the H37Rv DNA insert. Hybridizing cosmids (pYUB353 and pYUB354) was transformed into H37Ra. Randomly chosen, unrelated cosmids (pYUB355 and pYUB356) were transformed into H37Ra to serve as negative controls.

The H37Rv DNA of mc^{2}806 Confers In Vivo Growth Advantage to H37Ra

Table 2 summarizes the results of the growth behavior of the above-mentioned H37Rv transformants. The growth behavior of mc^{2}806 was mimicked by those H37Ra recombinants that contained pYUB352-overlapping cosmids; growth of the H37Ra recombinants containing the pYUB352-nonoverlapping cosmids was negligible. Clearly, the H37Ra DNA that is shared by pYUB352, pYUB353, and pYUB354 expresses a gene or gene(s) that confer on H37Ra the ability to grow in mouse spleen.

Mapping the *ivg* Region of H37Rv

The pYUB352, pYUB353, and pYUB354 cosmids were mapped by restriction digest and analyzed by Southern hybridization. A DNA region of ~25 kb is shared among the clones. This region was designated *ivg* (for in vivo growth advantage).

SUMMARY

The purpose of this work was to develop a system to identify virulence determinants of *M. tuberculosis* by genetic complementation. The ability to grow in mouse spleen and/or lung was defined as a potential phenotype for virulence. Enrichment for growing recombinant clones from a pool of H37Ra transformants containing the integrating pYUB178::H37Rv cosmid library was accomplished by in vivo selection. A molecular strategy was devised to isolate and clone the 25-kb H37Rv genomic fragment *ivg* that conferred in vivo growth advantage to H37Ra. This study is a first step toward understanding the genetics of virulence in *M. tuberculosis*. A detailed discription of these experiments has been submitted for publication in *Infection and Immunity*.

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