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Use of In Vivo Complementation in *Mycobacterium tuberculosis* To Identify a Genomic Fragment Associated with Virulence

LISA PASCOPELLA,[1]† FRANK M. COLLINS,[2]‡ JEAN M. MARTIN,[1] MONG HONG LEE,[3] GRAHAM F. HATFULL,[3] C. KENDALL STOVER,[4]§ BARRY R. BLOOM,[1] AND WILLIAM R. JACOBS, JR.[1]*

Department of Microbiology and Immunology, Howard Hughes Medical Institute, Albert Einstein College of Medicine, Bronx, New York 10461[1]; Trudeau Institute, Saranac Lake, New York 12983[2]; Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15260[3]; and MedImmune, Inc., Gaithersburg, Maryland 20878[4]

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Novel molecular tools and genetic methods were developed to isolate genomic fragments of *Mycobacterium tuberculosis* that may be associated with virulence. We sought to restore virulence, a characteristic of *M. tuberculosis* that is correlated with growth rate in mouse spleen and lung tissue, to the avirulent strain H37Ra by complementation. A representative library of the virulent *M. tuberculosis* strain H37Rv was constructed and transformed into H37Ra. Enrichment for individual faster-growing recombinants was achieved by passage of pools of H37Ra transformants harboring the H37Rv library through mice. A molecular strategy was devised to isolate and clone the H37Rv genomic DNA fragment *ivg*, which conferred a more rapid in vivo growth rate to H37Ra.

Tuberculosis is a worldwide health problem that causes approximately 3 million deaths each year (13); however, little is known about the molecular basis of the pathogenesis of tuberculosis. The disease is caused by infection with *Mycobacterium tuberculosis*; tubercle bacilli are inhaled and then ingested by alveolar macrophages. As is the case with most pathogens, infection with *M. tuberculosis* does not always result in disease. The infection is often arrested by a developing cell-mediated immunity resulting in the formation of microscopic lesions, or tubercles, in the lung. If cell-mediated immunity does not limit the spread of *M. tuberculosis*, caseous necrosis, bronchial wall erosion, and pulmonary cavitation may occur (5). The factors that determine whether infection with *M. tuberculosis* results in disease are incompletely understood.

The ability to transfer and express recombinant DNA among the mycobacteria, which has been recently demonstrated (11, 20), enables the usage of molecular genetics to elucidate pathogenic mechanisms. However, the present lack of evidence of homologous recombination in the pathogenic mycobacteria prevents the application of allele exchange systems (12). We sought to develop an in vivo genetic complementation system that utilizes integrating shuttle cosmid libraries to identify potential virulence genes of *M. tuberculosis*. One of the first examples of in vivo selection for virulent bacteria was demonstrated by the classic work of Griffith in 1928 (7). Griffith observed that as a result of genetic exchange between bacteria, virulent, capsulated pneumococci were recovered from mice infected with a mixture of live attenuated, noncapsulated pneumococci and heat-killed capsulated pneumococci (7).

A strategy to identify virulence determinants by genetic complementation requires (i) two strains that are genetically similar, (ii) a phenotype associated with virulence, and (iii) gene transfer systems. An existing pair of *M. tuberculosis* strains, H37Rv (virulent) and H37Ra (avirulent), distinguishable by their ability to cause disease in animal models (17), was used. H37Ra and H37Rv were derived from the same clinical isolate in 1934 (17, 21), and pulsed-field gel analyses of DNA fragments generated by digestion with infrequently cutting enzymes revealed that their macroscopic genome organizations are similar (1). Previous studies of H37Ra and H37Rv pathogenicity (4, 15, 16, 19) demonstrated the correlation of the extent of disease in animal models to the extent of multiplication in mouse spleen and lung tissue. We defined a potential virulence phenotype of recombinants as the ability to grow faster than H37Ra in mice.

A genomic library of *M. tuberculosis* H37Rv was constructed in an integrating cosmid vector, pYUB178, and electroporated into H37Ra. Mice were infected with pools of H37Ra recombinants containing H37Rv DNA to allow the selection of growing clones in mouse spleens and lungs. The integrating shuttle cosmid libraries are ideal for in vivo complementation because (i) theoretically, only 225 clones are required to represent the H37Rv genome, (ii) toxic effects associated with the expression of genes from multicopy plasmids are avoided, (iii) kanamycin selection pressure is not necessary to maintain the cosmid, and (iv) clusters of contiguous genes may be delivered and expressed.

The growth rates of selected recombinants in mouse spleens and lungs were measured, and a method to retrieve the H37Rv insert DNA from the chromosome of a recombinant was developed.

The strategy developed here was used to identify and partially characterize a 25-kb DNA fragment of *M. tuberculosis* which conferred an in vivo growth advantage to the growth-defective H37Ra.

* Corresponding author. Mailing address: Howard Hughes Medical Institute, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461 Phone: (718) 430-888. Fax: (718) 518-0366.

† Present address: Rocky Mountain Laboratories, National Institute of Allergy and Infectious Disease, National Institutes of Health, Hamilton, MT 59840.

‡ Present address: Division of Bacterial Products, Center for Biologics Evaluation and Research, FDA, Bethesda, MD 20892.

§ Present address: PathoGenesis Corporation, Seattle, WA 98119.

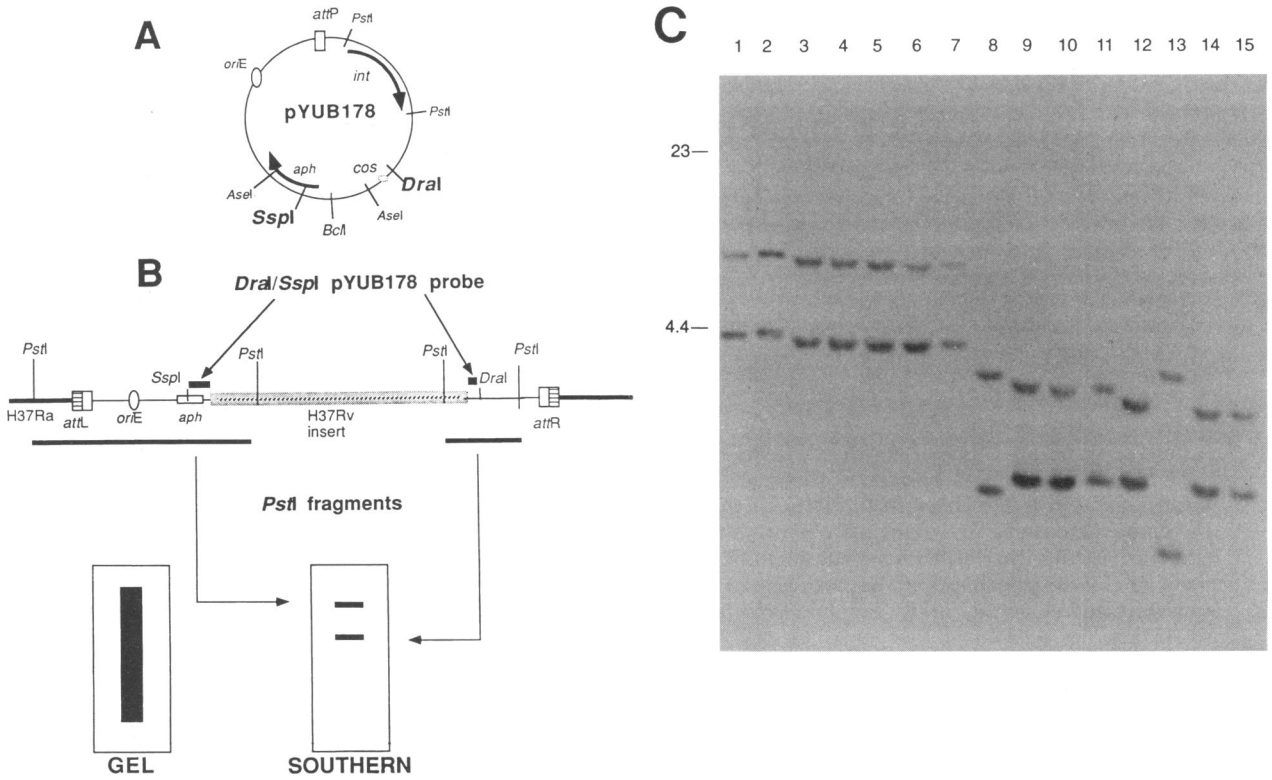

FIG. 1. Map of the integrating shuttle cosmid pYUB178 and analysis of individual clones and pools of H37Ra(pYUB178::H37Rv). (A) The components that allow integration of pYUB178 into the mycobacterial genomes are *attP* and *int* (13). The 5011-bp pYUB178 cosmid contains an *E. coli ori*, the L5 *attP*, the L5 *int*, a kanamycin resistance gene, *aph* (derived from Tn*903*), lambda *cos*, and a unique cloning site, *Bcl*I. (B) Schematic showing identification of the H37Rv DNA fragments that are adjoining pYUB178 sequences, i.e., junctional fragments, within the chromosome of an H37Ra recombinant containing pYUB178::H37Rv DNA. *Pst*I-digested chromosomal DNA was separated by gel electrophoresis and hybridized with a labelled probe from pYUB178. The probe was the 1.1-kb *Dra*I-*Ssp*I DNA fragment of pYUB178 that flanks the *Bcl*I cloning site. The integrated pYUB178::H37Rv cosmid can be detected only by the presence of pYUB178-hybridizing DNA fragments. The *Pst*I sites on either side of the H37Rv insert, near *attB* in H37Ra and near *cos* in pYUB178, are fixed. Thus, the sizes of hybridizing DNA fragments vary with the H37Rv insert DNA. (C) Junctional fragments of in vivo-selected H37Ra(pYUB178::H37Rv) recombinant clones. Individual H37Ra recombinants containing pYUB178::H37Rv cosmid clones were isolated from mouse lung tissue after passage of recombinant pools in spleens. The chromosomal DNAs from 15 clones were isolated, digested with *Pst*I, separated by agarose gel electrophoresis, and transferred to a nylon filter to hybridize with the 1.1-kb *Dra*I-*Ssp*I DNA fragment of pYUB178. Lanes 1 to 8, the H37Rv DNA junctional fragments of clones isolated from the H37Ra(pYUB178::H37Rv) pool 1; lanes 9 to 15, the H37Rv DNA junctional fragments of clones isolated from the H37Ra(pYUB178::H37Rv) pool 2. Note the presence of the same junctional fragments in many of the clones from the same pool. The clone mc^{2}806 in lane 12 was further characterized.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. tuberculosis* H37Ra and H37Rv were provided by Wilbur Jones of the Centers for Disease Control, Atlanta, Ga., and were grown in enriched 7H9 broth (Middlebrook 7H9 medium enriched with albumin-glucose complex or oleic acid-albumin-glucose complex [Difco Laboratories, Detroit, Mich.] and 0.05% polyoxyethylene sorbitan monooleate [Tween 80]) under Biosafety Level 3 containment. All cultures were grown at 37°C in tissue culture flasks without agitation. *Escherichia coli* χ2764 (9), HB101 (2), and DH5α (Bethesda Research Laboratories Life Technologies Inc., Gaithersburg, Md.) were grown with agitation in L broth. Strain χ2764 was grown at 30°C.

Construction of shuttle cosmid and H37Rv library. The pYUB178 integrating shuttle cosmid (Fig. 1A) was constructed by ligating the 975-bp *cos*-containing *Bgl*I-*Bcl*I fragment of lambda DNA to the *Bcl*I-digested, calf intestine alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.)-treated pMV305.F under conditions which favored the formation of linear concatamers, i.e., a final DNA concentration of greater than 50 ng/μl. The vector pMV305.F was a precursor to the vector pMV361 (22).

Genomic DNA of H37Rv was prepared by mechanical disruption of bacterial cells, which was accomplished by homogenizing a bacterial pellet in a tube with glass beads and buffer and by subsequent phenol-chloroform extractions (8). H37Rv genomic DNA was partially digested with a range of concentrations of *Sau*3A to generate 30- to 50-kb fragments. Fragments of 30 to 50 kb were electroeluted from agarose gels by a previously described procedure (10). The 30- to 50-kb *Sau*3A fragments of chromosomal DNA were then ligated to calf intestine alkaline phosphatase-treated, *Bcl*I-digested pYUB178 DNA; the final DNA concentration was 50 to 100 ng/μl, and the DNA molar ratio of insert to vector was 1.

Library packaging into lambda phage heads and tails. Four microliters of a 10-μl ligation mixture was in vitro packaged with the GigaPack II Packaging Extract (Stratagene, La Jolla, Calif.) according to the manufacturer's procedure. The in vitro-packaged lysate was transduced by previously described

methods (10) into the in vivo packaging strain of *E. coli* χ2764 (9).

In vivo packaging. The 10^3 to 10^4 kanamycin-resistant recombinant clones were pooled and inoculated into L broth containing 25 μg of kanamycin per ml. One aliquot was grown to prepare plasmid DNA by an alkaline lysis method. The other aliquot was grown for in vivo packaging that was accomplished by previously described procedures (9). The titer of the lysate prepared from χ2764 transductants containing the pYUB178::H37Rv library was approximately 10^9 CFU/ml. The lysate was stored at 4°C after filtering through a 0.45-μm-pore-size sterile filter.

Construction of H37Ra(pYUB178::H37Rv) recombinant pools. An 8-day-old H37Ra culture was electroporated with the pYUB178::H37Rv library DNA in plasmid form and then separately with pYUB178 DNA. Approximately 450 transformants arose from five independent electroporations of cells with approximately 1 μg of library DNA each. Two pools of H37Ra(pYUB178::H37Rv) recombinants (pool 1 and pool 2) were made by collecting and inoculating approximately 225 colonies into 50 ml of enriched 7H9 broth containing 10 μg of kanamycin per ml and by allowing growth for approximately 2 weeks. Aliquots of pools were distributed and frozen in cryovials for later use in animal experiments.

Mouse infection. Groups of C57BL/6 mice aged 6 to 8 weeks were intravenously inoculated with 0.2 ml of each culture tested. Five mice were inoculated with each recombinant group or control group per time point. Inoculation of mice with spleen-passaged bacteria was accomplished by first homogenizing the spleens after 14 days of infection in 5 ml of sterile saline. One milliliter of the 5-ml spleen homogenate 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 in experiments J2P and J5P. See Table 1 for details of mouse experiments.

Retrieval of pYUB178::H37Rv cosmids from chromosomes of in vivo-selected recombinants. Chromosomal DNA was isolated from individual H37Ra(pYUB178::H37Rv) recombinant clones by the method described by van Soolingen et al. (23). Briefly, bacterial pellets were treated in a stepwise fashion with lysozyme, proteinase K, and sodium dodecyl sulfate and were mixed with hexadecyltrimethylammonium bromide (Sigma Chemical Co., St. Louis, Mo.) and salt prior to extraction with chloroform. DNA was partially digested with *Sau*3A; fragments of 30 to 50 kb were size fractionated and eluted from agarose gels as described above. The 30- to 50-kb fragments were ligated to the 975-bp *Bgl*I-*Bcl*I fragment containing *cos* of coliphage lambda DNA. The ligation conditions were such that the final DNA concentration was 50 to 100 ng/μl, and the molar ratio of chromosomal DNA fragments to *cos* DNA fragments was 1.

The ligation mixture was packaged into lambda phage heads and tails by using the Stratagene GigaPack kit and transduced into *E. coli* HB101. Individual kanamycin-resistant transductant colonies were picked, and cosmid DNA was isolated. Cosmid DNA was then analyzed by restriction digestion and Southern hybridization.

Restriction and Southern analyses of retrieved cosmids. Digested cosmid DNA was subjected to agarose gel electrophoresis in 0.8% agarose in Tris-acetate buffer. DNA was Southern blotted from gels onto nylon membranes by capillary diffusion, UV cross-linked, and hybridized with probes derived from pYUB178. Probes consisted of either the 1.1-kb *Dra*I-*Ssp*I fragment of pYUB178 or the 434-bp *Ase*I-*Bcl*I fragment of pYUB178 that contained lambda DNA adjacent to *cos* or the 756-bp *Ase*I-*Bcl*I fragment of pYUB178 that contained part of *aph*. Probes were labelled with [α-^{32}P]dCTP by using random hexamer priming with the Pharmacia oligolabelling kit (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) or with the Enhanced Chemiluminescence ECL Gene Detection system (Amersham International, Amersham, United Kingdom).

Screening the pYUB178::H37Rv library in *E. coli*. The pYUB178::H37Rv library DNA lysate (10^9 CFU/ml) was serially diluted to a concentration of 10^4 CFU/ml in SM buffer (50 mM Tris-Cl [pH 7.5], 100 mM NaCl, 8 mM $MgSO_4 \cdot 7H_2O$) and transduced into *E. coli* HB101. Aliquots of infected cells were plated onto L agar containing 25 μg of kanamycin per ml such that each plate would contain approximately 150 colonies. After overnight incubation at 37°C, colonies from each plate were lifted onto Biotrans nylon filters (ICN Biomedicals, Inc., Irvine, Calif.) and prepared according to the protocol of the manufacturer (ICN Biomedicals, Inc.) for hybridization. A probe was made from a cosmid, pYUB352, derived from the mc^{2}806 recombinant clone. The cosmid pYUB352 was linearized by digestion with *Ase*I and labelled with [α-^{32}P]dCTP. Filters were hybridized overnight according to the protocol of the manufacturer (ICN Biomedicals, Inc.).

RESULTS

Construction of shuttle cosmid and H37Rv library. The integrating cosmid pYUB178 contains an *E. coli ori*-derived from pUC19, the L5 *attP* site, the L5 integrase gene, a kanamycin resistance gene, *aph*, derived from Tn*903*, the lambda *cos* sequence, and a unique cloning site, *Bcl*I (Fig. 1A). The L5 mycobacteriophage attachment site (*attP*) and integrase gene (*int*) mediate site-specific integration into the *attB* site of the mycobacterial chromosome (14). The H37Rv library was constructed by ligating 40-kb size-selected chromosomal DNA fragments, generated by partial digestion with *Sau*3A, to alkaline phosphatase-treated pYUB178, which was linearized by digestion with *Bcl*I. The ligation mix was packaged into lambda phage heads and tails and transduced into *E. coli*. The approximately 4,000 kanamycin-resistant transductant colonies were theoretically enough to represent the H37Rv genome 18 times. Twelve individual cosmids of the H37Rv library were isolated from randomly picked *E. coli* transductant colonies and examined by restriction analyses. No two cosmids were alike, and each cosmid had an insert size of 35 to 40 kb (data not shown), suggesting that the library was representative.

The H37Rv library DNA was isolated as plasmid from the complete pool of *E. coli* transductants and electroporated into H37Ra. To identify the H37Rv insert within the chromosome of a H37Ra(pYUB178::H37Rv) recombinant, junctional fragment analysis, depicted in Fig. 1B, was devised. *Pst*I restriction fragments that include H37Rv DNA adjacent to pYUB178 sequences on either side of the *Bcl*I cloning site are visualized by Southern hybridization (Fig. 1B) with a pYUB178 probe.

Enrichment and selection of putatively virulent recombinants from pools. Mice were intravenously infected with either H37Ra(pYUB178::H37Rv) recombinant pool 1 or pool 2. Recombinants that either grew or persisted in mouse spleens for 2 weeks were inoculated into a second group of mice (Table 1 [experiment J5P]). Forty colonies were recovered from the plated, homogenized lungs of these mice as follows: 14 colonies from pool 1 and 26 colonies from pool 2. Junctional fragment analyses of 16 selected individual recombinants are shown in Fig. 1C. The fact that eight different clonal types represent 19 individual recombinants (only 15 are shown; 4

TABLE 1. Details of mouse infections

Expt	Pool(s) and/or clone(s) tested	Inocula (CFU/mouse)	Time points (days)
J2	Pool 1	2×10^5	
	Pool 2	6×10^5	1, 14, 28
	mc^{2}816	1×10^6	
J5	Pool 1	1×10^5	
	Pool 2	6×10^5	1, 14, 28
	mc^{2}816	1×10^6	
	H37Rv	6×10^4	
J2P[a]	Pool 1	5×10^2	
	Pool 2	7×10^2	1, 14
	mc^{2}816	5×10^2	
J5P[a]	Pool 1	9×10^2	
	Pool 2	7×10^2	1, 14
	mc^{2}816	6×10^3	
J33	mc^{2}806	1×10^4 to 2×10^4	
	mc^{2}816	4×10^4	1, 14, 28, 84
	H37Rv	5×10^4	
J36	mc^{2}806	1×10^4	
	mc^{2}822	1×10^4 to 2×10^4	
	mc^{2}823	1×10^4 to 3×10^4	
	mc^{2}824	5×10^4	2, 14, 28, 87
	mc^{2}825	6×10^4	
	mc^{2}816	8×10^4	
	H37Rv	4×10^4	

[a] Inocula were estimated from CFU retained in the spleen on day 1; spleen retention is usually 10% of the inoculating dose.

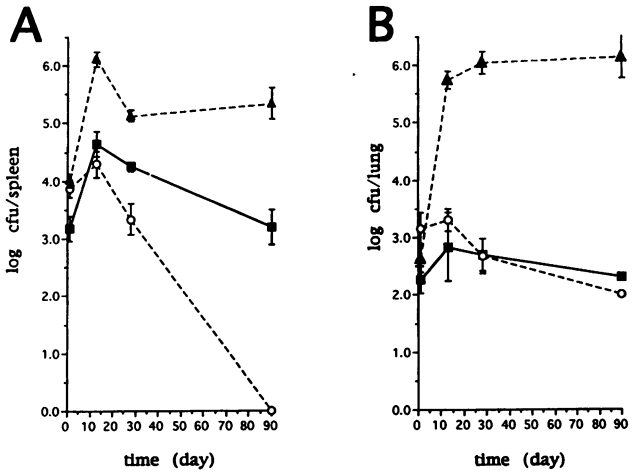

FIG. 2. Growth of mc^{2}806 in mouse lung and spleen tissues. The growth rates of clones mc^{2}806, H37Rv, and mc^{2}816 were measured and compared. The growth rate of mc^{2}806 is represented by solid squares on the solid lines, the growth rate of mc^{2}816 is represented by the open circles on the dotted lines, and the growth rate of H37Rv is represented by solid triangles on the dotted lines. These data are from a single experiment that is representative of three experiments. See text and Table 1 (experiment J33) for experimental details. (A) Growth in spleen; (B) growth in lung.

more clones were identical to those in lane 12) from both pools suggests that in vivo passage selected for clones having particular genotypes. Because clones having junctional fragments identical to those of mc^{2}806 (Fig. 1C, lane 12) were isolated from many animals during two different experiments (J2P and J5P [data not shown]), mc^{2}806 was further characterized. Its in vivo growth rate was intermediate to the growth rates of mc^{2}816 (H37Ra containing pYUB178) and H37Rv (Fig. 2). Notably, the growth rates of mc^{2}806, mc^{2}816, and H37Rv in enriched 7H9 broth were virtually identical (data not shown).

Retrieval of a H37Rv DNA insert that confers a faster in vivo growth rate to H37Ra. To prove that the H37Rv DNA insert present in mc^{2}806 was responsible for its in vivo growth phenotype, it had to be retrieved from the chromosome. A disadvantage of the stably integrating pYUB178::H37Rv cosmid library is the difficulty of cosmid retrieval from the chromosome of an H37Ra(pYUB178::H37Rv) recombinant; the excision functions of L5 are not yet understood. Hence, a method was devised to clone the H37Rv DNA insert as a cosmid (Fig. 3A). The lambda in vitro-packaged ligation mix, which contained random pieces of the mc^{2}806 chromosome, was transduced into *E. coli* for the purpose of selecting H37Rv DNA-containing cosmids. Only cosmids containing the *E. coli ori* and *aph* genes replicated under kanamycin selection pressure (Fig. 3A). To determine whether the entire H37Rv insert DNA was retrieved, cosmid junctional fragments, which were generated by digestion with *Eco*RI and *Ase*I, were compared with mc^{2}806 junctional fragments. Southern analyses were performed sequentially with two probes, which were generated by digestion of pYUB178 with *Ase*I and *Bcl*I; the 434-bp probe hybridizes to the DNA fragment containing H37Rv adjacent to *cos*, and the 756-bp probe hybridizes to the DNA fragment containing H37Rv adjacent to *aph* (Fig. 3A). All 33 retrieved cosmids had the same 6-kb *aph* side junctional fragment as mc^{2}806 (data not shown), and only 1 of the retrieved cosmids did not have the 2.1-kb *cos* side junctional fragment (Fig. 3B, lane 14). The cosmid designated pYUB352 in lane 15 was used for further study.

Identification of pYUB352-overlapping cosmids from the pYUB178::H37Rv DNA library. Retrieved cosmids *did not* have the ability to integrate into mycobacteria because they lost the *int* gene when they were removed from the chromosomes of the recombinants. Therefore, pYUB352 DNA was used as a probe to screen the pYUB178::H37Rv library in *E. coli* for the H37Rv DNA insert associated with mc^{2}806. Colonies of *E. coli*(pYUB178::H37Rv) library transductants were transferred to nylon filters, lysed, and probed with pYUB352 DNA. Of the approximately 500 colonies screened, 4 clones contained H37Rv DNA inserts that hybridized to the H37Rv DNA insert of pYUB352 (data not shown). Cosmids that shared H37Rv DNA with pYUB352 (designated pYUB353 and pYUB354) and unrelated cosmids (designated pYUB355 and pYUB356) were separately transformed into H37Ra.

The H37Rv DNA of mc^{2}806 confers in vivo growth advantage to H37Ra. The growth rates of H37Ra recombinants containing pYUB352-overlapping and -nonoverlapping cosmids were tested in mice (Table 1, experiment J36). The H37Ra recombinants containing the pYUB352-overlapping cosmids grew as well as mc^{2}806, and the H37Ra recombinants containing pYUB352-nonoverlapping cosmids grew poorly or did not grow at all (Fig. 4). These data indicate that the H37Rv DNA that is shared by pYUB352, pYUB353, and pYUB354 expresses a gene or gene(s) associated with growth in the spleen.

Mapping the *ivg* region of H37Rv. The pYUB352, pYUB353, and pYUB354 cosmids were mapped by restriction digestion and were analyzed by Southern hybridization (Fig. 5). The schematic of Fig. 5C shows the physical map of the

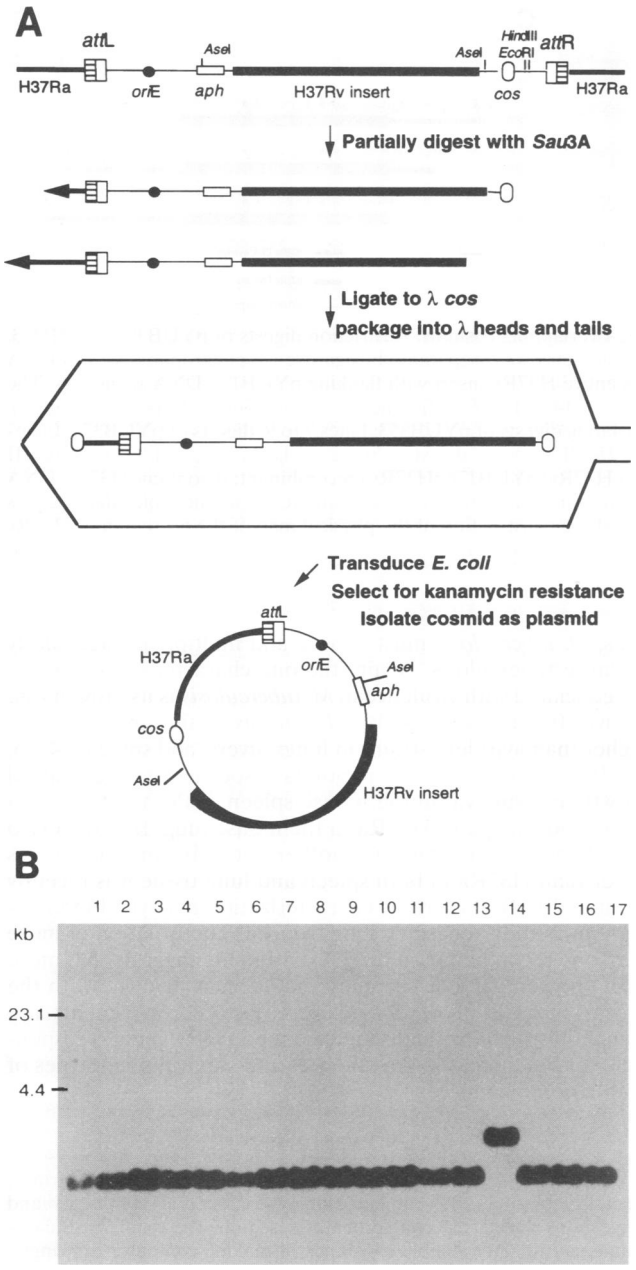

FIG. 3. Retrieval of H37Rv-containing cosmids from the mc^{2}806 chromosome. (A) A schematic illustrating the strategy used to retrieve the H37Rv insert DNA from the integrated cosmids in H37Ra (pYUB178::H37Rv) recombinants; (B) Southern hybridization of *Ase*I and *Eco*RI digests of mc^{2}806 chromosomal DNA, or cosmid DNAs that were retrieved from the chromosome of mc^{2}806. The 434-bp *Ase*I-*Bcl*I fragment of pYUB178 that contained *cos* was used as a probe. Lane 1, mc^{2}806 chromosomal DNA; lanes 2 to 17, DNA from 16 individual retrieved cosmids.

H37Rv DNA insert of each clone. In addition, both *Pst*I and *Sac*I restriction digests revealed at least 20 kb of overlap between the H37Rv inserts of pYUB352, pYUB353, and pYUB354 (data not shown). The 25 kb of shared DNA depicted in Fig. 5C was designated *ivg* for in vivo growth advantage.

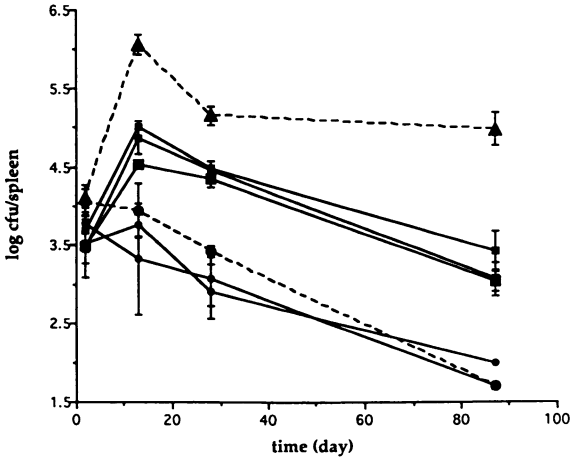

FIG. 4. The growth of H37Ra recombinants containing pYUB352-overlapping and -nonoverlapping cosmids. H37Ra was separately transformed with pYUB352-overlapping cosmids (pYUB353 and pYUB354) and with unrelated cosmids (pYUB355 and pYUB356). The growth of each recombinant was measured over a time course in mouse spleens (see Table 1 [experiment J36]). The growth of pYUB353- and pYUB354-containing H37Ra recombinants is represented by the small squares on the solid lines. The growth of mc^{2}806 is represented by the large squares on the solid lines. The growth of pYUB355- and pYUB356-containing H37Ra recombinants is represented by the small circles on the solid lines. The growth of mc^{2}816 is represented by the large circles on the dotted lines. The growth of H37Rv is represented by the triangles on the dotted lines.

DISCUSSION

In the absence of a system for allelic exchange, complementation analysis provides a means to identify genes associated with virulence. Although various phenotypes of bacterial pathogens may be associated with pathogenicity, the only characteristic of *M. tuberculosis* that has been shown to be correlated with virulence is the ability to survive and grow in vivo (4, 15, 16, 19). The identification of complementing genes from a genomic DNA library by screening for growth in an animal poses unique challenges. Since the number of clones that are screened should be minimized, cosmid genomic libraries offer one way to accomplish this end. However, cosmid clones can be unstable, particularly when the bacterial host is grown in vivo. The expression of multiple copies of particular genes may adversely affect the growth of the bacterial host and thus result in the loss of specific sequences or the entire cosmid from the library. Also, extrachromosomal cosmids may not be useful for in vivo screening because they can be inefficiently replicated and/or partitioned when the mycobacterial host is grown in vivo (18a). Integrating shuttle cosmid libraries that replicate as multicopy plasmids in *E. coli* and integrate into the mycobacterial chromosome remedy these limitations for mycobacterial hosts. First, since mycobacterial genes are generally not well expressed in *E. coli*, library bias resulting from instability caused by multicopy expression during amplification in *E. coli* is minimized. Second, the use of the L5 integration system enhances the stability of the cosmid in the mycobacterial host. Mycobacterial genes that are expressed from a cosmid integrated in *attB* are especially stable because they are present in single copy; the propagation of the integrating library in mycobacteria does not generally result in loss of representation. In addition, individual cosmids integrate into a single known chromosomal site and therefore do not disrupt mycobacterial host genes, and they are stable in the

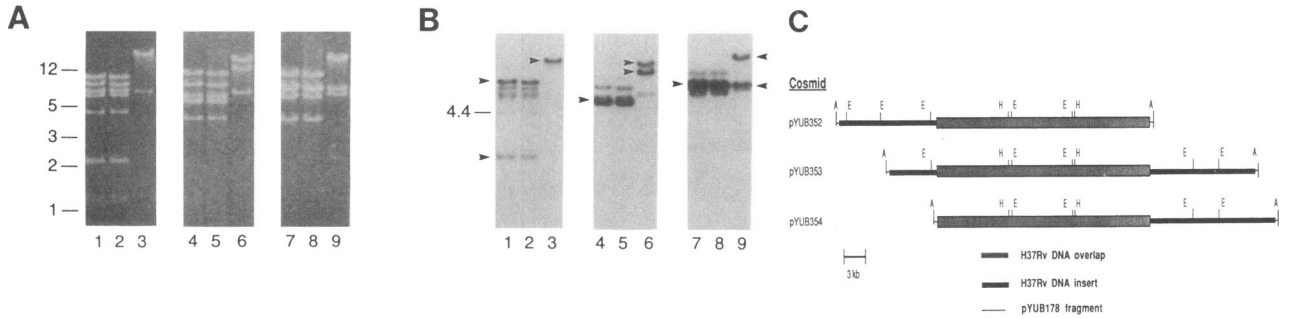

FIG. 5. Restriction mapping of the *ivg* region of H37Rv DNA in pYUB352-overlapping cosmids. Restriction digests of pYUB352, pYUB353, and pYUB354 were performed with *Eco*RI and *Hin*dIII. (A) Digested DNA fragments were separated by agarose gel electrophoresis. (B) DNA fragments were hybridized to the *Ase*I fragment of pYUB352 that included its entire H37Rv insert with flanking pYUB178 DNA sequences. The arrows point to DNA fragments that hybridize to pYUB178 DNA probes (the 434-bp *Ase*I-*Bcl*I fragment and the 756-bp *Ase*I-*Bcl*I fragment). These bands are junctional fragments. Lanes 1 to 3, digests of pYUB352; lanes 4 to 6, digests of pYUB353; lanes 7 to 9, digests of pYUB354. Lanes 1, 4, and 7 show *Eco*RI digestion patterns; lanes 2, 5, and 8 show *Eco*RI and *Hin*dIII double digestion patterns; lanes 3, 6, and 9 show *Hin*dIII digestion patterns. Note that pYUB352 was derived from the chromosome of a H37Ra(pYUB178::H37Rv) recombinant; it contains H37Ra DNA adjacent to *attB* and sequences of pYUB178 DNA that span the *aph* gene and *attP*, including *oriE*. (C) Data gathered from the molecular analyses shown in Fig. 5A and Fig. 5B and the functional analyses shown in Fig. 4 allowed the construction of the physical map of the *ivg* region of H37Rv that is present in cosmids pYUB352, pYUB353, and pYUB354. A, *Ase*I; E, *Eco*RI; H, *Hin*dIII.

absence of selective pressure (14). The identification of *ivg*, a genomic region of the virulent H37Rv that confers enhanced in vivo growth to the avirulent H37Ra, demonstrates that integrating shuttle cosmid libraries are useful tools for genetic screening in vivo.

Although a genomic region associated with in vivo growth was identified, full restoration of growth was not accomplished. There are three possible explanations for the inability to restore full virulence or the maximal in vivo growth rate of H37Rv to H37Ra with the shuttle cosmid library. One is that the H37Ra strain used in this study likely has multiple mutations in noncontiguous chromosomal loci. The source of H37Ra is a stock strain that has been passaged through liquid media for the past 60 years. Repeated passage of *M. tuberculosis* results in the selection of attenuated variants that have most likely accumulated many mutations. In fact, the attenuated vaccine strain *Mycobacterium bovis* BCG was isolated by Calmette and Guerin after repeated in vitro passage (3). Perhaps complementation of the H37Ra recombinants containing the H37Rv *ivg* locus with a second integrating cosmid library of H37Rv would restore full virulence. Another explanation for incomplete restoration of the H37Rv growth phenotype in the recombinants is that the complementing gene(s) of H37Rv is not fully expressed in or is inhibited by the presence of the mutated allele(s) in H37Ra. Epistatic interactions of H37Ra and H37Rv alleles within a H37Ra (pYUB178::H37Rv) recombinant may prevent full expression of the complementing gene(s). Testing whether the expression of a mutated H37Ra gene is dominant to that of a wild-type gene will have to await the development of methods to inactivate targeted genes in *M. tuberculosis*. Another possible limitation of this approach is that the gene(s) required to restore full virulence was not represented in the pool of recombinants containing the H37Rv integrating shuttle cosmid library. Although we have shown that the library appears to be representative and stable in *E. coli*, we cannot rule out the possibility that particular *M. tuberculosis* DNA fragments are inherently unstable either in *E. coli* or in H37Ra.

Microbial pathogens must undergo a series of steps (adherence and/or entry, multiplication, immune evasion, and dissemination) to establish an infection and/or cause disease (6). As a member of the group of facultative intracellular pathogens, *M. tuberculosis* must survive and multiply intracellularly to cause tuberculosis. Again, the one characteristic known to be associated with virulence in *M. tuberculosis* is its growth rate in vivo. In mice, virulent *M. tuberculosis* strains grow at a rate higher than avirulent strains in lungs, livers, and spleens (4, 15, 16, 19). Although the *ivg* region is associated with enhanced growth and survival in the mouse spleen, it does not confer a growth advantage to H37Ra in the mouse lung. By the in vivo complementation strategy, another recombinant that grows faster than H37Ra in both spleen and lung tissue was recently identified (18). Its further characterization may yield interesting information regarding lung tropism. The isolation of more than one recombinant in these experiments suggests that more than one gene and/or chromosomal locus is involved with the in vivo growth of *M. tuberculosis*. Future experiments are planned to subclone and sequence the H37Rv gene(s) responsible for the increased growth rates and survival phenotypes of both recombinants.

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