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Escherichia coli-Mycobacteria Shuttle Vectors for Operon and Gene Fusions to *lacZ*: the pJEM Series

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A series of *Escherichia coli*-mycobacteria shuttle plasmids for the isolation and study of gene regulatory sequences was constructed. These pJEM vectors contain an efficient transcription terminator and multiple cloning sites and allow either operon or gene fusions to *lacZ*. By constructing operon fusions with pJEM15, we assessed various previously characterized mycobacterial promoters in the fast-growing species *Mycobacterium smegmatis* and the slow-growing species *M. bovis* BCG. Our results suggest that *M. smegmatis* and *M. bovis* BCG RNA polymerases do not share the same specificity. To isolate new mycobacterial promoters, an *M. tuberculosis* DNA library was generated, using pJEM13, and screened in *M. smegmatis*. Several Lac$^+$ clones were isolated, and the β-galactosidase activity was measured.

The genus *Mycobacterium* includes major human pathogens such as *Mycobacterium leprae* and *M. tuberculosis*, the causative agents of leprosy and tuberculosis, which remain serious worldwide health problems. The avirulent strain of *M. bovis* which has been extensively used as a tuberculosis vaccine, BCG (bacille Calmette-Guérin), is also a very attractive vector for the construction of live recombinant vaccines particularly because of its strong immunogenicity. As a result, there is currently much interest in studying the molecular biology of mycobacteria.

An important aspect of this research is the construction of genetic tools for studying gene expression in mycobacteria. Regulatory sequence-probe vectors have been extensively used to isolate and analyze regulatory sequences in many bacteria (15). The application of such tools to mycobacteria would facilitate the study of genetic mechanisms regulating virulence in pathogenic species and the isolation of new regulatory sequences which may be useful for the development of improved recombinant BCG vaccines.

Initially, mycobacterial gene expression was studied in heterologous systems, *Escherichia coli* and *Streptomyces lividans* (5, 12, 22). These analyses suggested that most mycobacterial genes are more efficiently expressed in *S. lividans* than in *E. coli*. Subsequently, mycobacterial plasmid-based vectors which could be used for studies in homologous systems were constructed. Vectors pYUB75 and pYUB76 were designed to select gene fusions to a truncated *E. coli lacZ* gene (1). Plasmid pSD7 allows the construction of operon fusions to a promoterless chloramphenicol acetyltransferase gene (6). With these vectors, a number of mycobacterial regulatory sequences were isolated and assessed in both *E. coli* and in *Mycobacterium smegmatis*.

In this report, we describe the construction of the pJEM series of vectors, which have several advantages: they carry a transcription terminator and convenient multiple cloning sites (MCSs) and allow both operon and gene fusions to *lacZ*. *lacZ* was chosen as the reporter gene because the enzyme encoded, β-galactosidase, remains active when heterologous sequences are fused to its amino terminus (4) (26). Its activity can be easily measured in vitro, even at very low levels, using fluorescent compounds (7). β-Galactosidase is also highly immunogenic. It elicits both humoral and cellular immune responses when presented to the immune system of mice by recombinant bacteria (3, 17). Thus, β-galactosidase may also be used as a reporter of the immunogenicity of a recombinant vaccine. By using pJEM vectors, new regulatory sequences active in BCG could be isolated and the recombinant BCG strains could be readily tested for the ability to induce immune responses in mice.

We illustrate the use of the vector allowing operon fusions, pJEM15, in a comparative study of activities of various previously characterized mycobacterial promoters in *M. smegmatis* and BCG. The utility of the vectors allowing gene fusions was shown by cloning and assessing gene regulatory sequences from an *M. tuberculosis* DNA library in pJEM13.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *M. smegmatis* mc^{2}155 (20) and *M. bovis* BCG Pasteur 1173P2 (BCG Laboratory Collection, Pasteur Institute) were used as mycobacterial hosts. *E. coli* MC1061 [F′ *araD139* Δ(*ara leu*)*769* Δ*lacX*74 *galU galK hsdR mcrB rpsL* thi] (4) and TGE901 λ cI857 Δ*H*1 *bio*) (Transgène) were used for plasmid preparation. Plasmids harboring the p_L promoter were propagated only in TGE901 cells grown at 30°C to avoid thermoinactivation of the *c*I857 repressor. Transformants of *M. smegmatis* and *E. coli* were selected on LB plates containing either kanamycin (25 μg/ml) or streptomycin (10 μg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 0.001%) as appropriate. BCG transformants were plated on 7H10 (Difco) supplemented with OADC (Difco), kanamycin (25 μg/ml), and X-Gal (0.001%). For β-galactosidase assays, *M. smegmatis* and BCG were grown in 7H9 (Difco) medium supplemented with ADC (Difco) and Tween 80 (0.05% for *M. smegmatis* and 0.1% for BCG). Cultures were incubated at 37°C with agitation until saturation, then diluted 50-fold in fresh medium, and incubated until reaching a optical density at 600 nm of approximately 0.8.

Plasmids and transformation. Plasmid pHP45Ω, which contains the Ω interposon, plasmid pRR3, and the pNM480 series have been described elsewhere (16, 19, 20). pJN1 (17) is a

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derivative of pTG959 (10) (Transgène) which harbors a *c*II-*lacZ* gene fusion under the control of the p_L promoter and a synthetic ribosome binding site (sRBS) (sequence shown in Fig. 2). The isolation of pJN3 and pJN11 is described in Fig. 1 and in the text. pJEM15 resulted from cloning into the pRR3 *Sca*I site (i) a fragment obtained by PCR amplification (using OJN1 [5′-AAGCTTCCGATTCGTAGAGCC-3′] and OJN2 [5′-GGGCTCGAGCTGCAGTGGATGACCTTTTGA-3′] as primers and pJN11 as the template) containing the transcription terminator of coliphage T4 (*t*T4) and the N terminus of *c*II, (ii) the synthetic oligonucleotides corresponding to MCS1, and (iii) the *Hin*dIII-*Dra*I *lacZ′* fragment from pNM480. pJEM12 to -14 were obtained by cloning the PCR-amplified fragment described above into the *Sca*I site in pRR3. The synthetic oligonucleotides corresponding to MCS2 were then inserted. Finally, each of the three forms of the pNM480 series was introduced into the *Hin*dIII site in MCS2. The fragments containing *p*blaF* (25), *p*AN (17), and *p*groES/EL1 (27) were obtained as previously described by in vitro PCR amplification and cloned into the MCS1 in pJEM15 to give pJN30, pJN31, and pJN32, respectively. The *p*sul3 promoter was isolated as a 344-bp *Bam*HI-*Kpn*I fragment of pIPC1 (13) and similarly cloned into pJEM15 to give pJN32. Bacteria were transformed by electroporation as described elsewhere (9). Electroduction between *M. smegmatis* and *E. coli* was performed by the method of Baulard et al. (2) at 200 Ω, 2,500 V, and 25 μF, using a Gene Pulser (Bio-Rad).

DNA manipulation and construction of the *M. tuberculosis* gene library. Standard recombinant DNA techniques were carried out as described previously (21). The genomic DNA library of *M. tuberculosis* isolate 103 (obtained from a tuberculous patient; laboratory collection) was produced by cloning partially *Sau*3AI-digested DNA (size, 0.2 to 2 kbp; purified from a sucrose gradient) into pJEM13 linearized with *Bam*HI and dephosphorylated with bacterial alkaline phosphatase. The resulting library was composed of approximately 1,200 *E. coli* clones containing recombinant plasmids. After amplification on LB–X-Gal plates, the clones were pooled and plasmid was prepared. The resulting plasmid library was then used to electroporate *M. smegmatis*.

β-Galactosidase assay. β-Galactosidase activity was assayed in sonicated extracts of *M. smegmatis* and BCG clones by the method of Pardee et al. (18). Experiments were carried out in triplicate. Units of β-galactosidase were calculated by the following formula: 200 × optical density at 420 nm per milligrams of protein per minute. Soluble protein in bacterial extracts was measured by the Bio-Rad assay.

RESULTS AND DISCUSSION

Construction of pJEM vectors. Ideally, a promoter-probe plasmid vector should contain five elements: (i) a replicon, (ii) a selectable marker, and a reporter cassette containing (iii) a transcription terminator followed by (iv) MCSs and (v) a reporter gene devoid of its regulatory sequences.

To construct a promoter-probe plasmid for mycobacteria, we used the *Mycobacterium fortuitum* plasmid pAL5000 replicon and the kanamycin resistance gene (*aph*) from Tn*903* (20). These genetic elements are the basic components of most plasmids currently used for transformation of mycobacteria. They appear to confer a high stability to transformed *M. smegmatis* and *M. bovis* BCG clones both in vitro and in vivo (in mice) even in the absence of drug selection (17). To facilitate the preparation and manipulation of episomal DNA, most of these plasmids also contain an *E. coli* replicon. Thus, we chose the pRR3 backbone, an *E. coli*-mycobacteria shuttle vector which contains these three genetic elements (20).

No mycobacterial transcription terminator has yet been characterized. To examine whether *t*T4 was active as a termination site for mycobacterial RNA polymerases, the omega interposon (Ω) (19) was cloned into plasmid pJN3, upstream from the sRBS-*c*II-*lacZ* element, generating pJN11 (Fig. 1). The Ω fragment is composed of a streptomycin-spectinomycin resistance gene flanked by short inverted repeats containing *t*T4. Insertion of Ω into a DNA fragment leads to termination of RNA synthesis in *E. coli* (19). pJN3 was constructed by cloning into the *Sca*I site of pRR3 a cassette composed of a truncated *lacZ* associated to an sRBS and the 5′ extremity of the lambda phage regulatory gene *c*II and p_L promoter (Fig. 1). *M. smegmatis* mc^2155 was transformed with pJN3 (p_L-sRBS-*c*II-*lacZ*) or pJN11 (p_L-Ω-sRBS-*c*II-*lacZ*) by electroporation and plated on LB–X-Gal plates. pJN3 transformants gave blue colonies, and pJN11 transformants gave white colonies. The β-galactosidase activity in *M. smegmatis*(pJN11) was 50 times lower than that in *M. smegmatis*(pJN3) (Table 1). Thus, *t*T4 in the Ω insert is an efficient transcription terminator in *M. smegmatis*.

A DNA fragment containing the *t*T4 segment followed by the sRBS-*c*II-*lacZ* element from pJN11 was synthesized in vitro by PCR amplification. An MCS (MCS1) containing six unique restriction sites was added. The resulting cassette was then cloned into the *Sca*I site of pRR3, giving the operon fusion vector pJEM15 (Fig. 2). Electroporation of *M. smegmatis* and BCG with this plasmid resulted in white colonies on LB–kanamycin–X-Gal plates with a very low β-galactosidase activity (Table 1). In contrast, *E. coli*(pJEM15) expressed higher β-galactosidase activity and consequently was blue on LB–X-Gal plates (data not shown). This is probably due to its high copy number. In *E. coli*, pUC vectors are present at a high copy number (over 500), where as in mycobacteria, pAL5000 replicon-derived plasmids have a copy number of approximately 3 to 10 (11). Testing DNA fragments for promoter activity, using pJEM15, by blue-white screening should thus be carried out directly in mycobacteria.

To obtain vectors allowing gene fusions to *lacZ*, we followed a similar strategy. The three forms of truncated *lacZ* from the pNM480 series (16), which differ from each other in the translation phasing of a *Hin*dIII site located at its 5′ extremity, were cloned downstream of *t*T4, and an MCS (MCS2) containing seven unique restriction sites was inserted into the *Sca*I site of pRR3. The resulting plasmids, pJEM12 to -14 (Fig. 2), thus allow the cloning of a wide range of restriction fragments in frame to *lacZ*.

Assessment of various promoters in *M. smegmatis* and BCG. Operon fusions between the *c*II-*lacZ* reporter cassette of pJEM15 and the promoters *p*AN (17), *p*blaF* (25), *p*sul3 (13), and *p*groES/EL1 (8) (27) were constructed. The activities of these promoters were assessed in the fast-growing *M. smegmatis* and in the slow-growing *M. bovis* BCG. The first three promoters were isolated from mycobacterial species: *p*blaF* is an up mutant of *p*blaF, which drives the expression of the *M. fortuitum* β-lactamase gene; *p*AN and *p*sul3 are components of mobile genetic elements, *M. paratuberculosis* IS*900* and *M. fortuitum* Tn*610*, respectively. These promoters have been located on the basis of transcription start site mapping (*p*blaF* and *p*AN) or by deletion analysis (*p*sul3) (24). *p*groES/EL1 is a promoter from *Streptomyces albus* which regulates the expression of the *groES/EL1* operon and is active in both *M. smegmatis* and BCG (27).

The cloning experiments were performed directly in *M. smegmatis*. DNA fragments containing one of each promoter

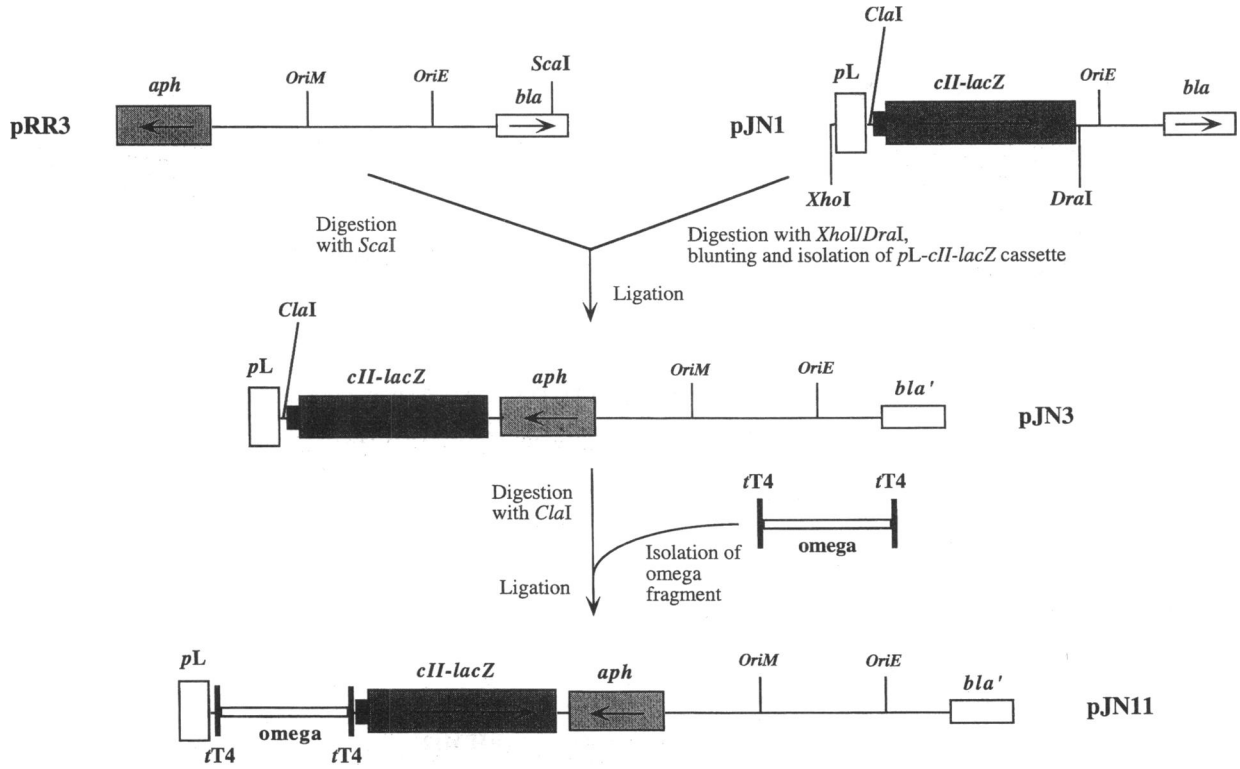

FIG. 1. Construction of plasmids pJN3 and pJN11. Only the relevant restriction sites and genetic elements are shown. In pJN3 and pJN11, the β-lactamase gene (*bla*) was disrupted. *oriE* and *oriM* designate the pUC (*E. coli*) and pAL5000 (mycobacteria) origins of replication, respectively.

were isolated and ligated to pJEM15 MCS1 digested with the appropriate restriction enzymes. The resulting ligation mixtures were used to transform *M. smegmatis* by electroporation, and blue colonies were selected to electroduce *E. coli* MC1061. Plasmids were isolated from these *E. coli* clones and analyzed. Those corresponding to the desired constructions (pJN29, pJN30, pJN31, and pJN32; Table 1) were used to electroporate BCG.

β-Galactosidase activity was assayed in sonicated extracts of *M. smegmatis* and BCG (Table 1). The activities of the promoters varied considerably both between promoters in one mycobacterial host and between hosts for each promoter. The relative strengths of the promoters was not the same in *M. smegmatis* and BCG. However, *p*blaF* was the most powerful promoter in both *M. smegmatis* and BCG. *p*AN and *p*groES/EL1 were more active than *p*sul3 in BCG, but in *M. smegmatis*, *p*sul3 was more active than *p*AN or *p*groES/EL1.

TABLE 1. β-Galactosidase activities of *M. smegmatis* and BCG clones expressing the various fusions to *cII-lacZ*

Plasmid (promoter)	β-Galactosidase activity[a] (U)	
	M. smegmatis	*M. bovis* BCG
pJN3 (p_L)	206 ± 48	ND[b]
pJN11 (p_L + Ω)	4 ± 3	ND
pJEM15	12 ± 6	35 ± 4
pJN29 (*p*sul3)	338 ± 10	264 ± 4
pJN30 (*p*blaF*)	2,923 ± 325	2,224 ± 337
pJN31 (*p*AN)	163 ± 51	364 ± 48
pJN32 (*p*groES/EL1)	299 ± 45	729 ± 26

[a] Results are means and standard deviations for three independent experiments.
[b] ND, not determined.

Das Gupta and collaborators (6) screened *M. smegmatis* and *M. tuberculosis* DNA libraries for promoter activity in *M. smegmatis*. They reported a 10- to 20-times-higher frequency of promoters in *M. smegmatis* DNA. Moreover, very active promoters were rarer in *M. tuberculosis* than *M. smegmatis* DNA libraries. They suggested that *M. tuberculosis* promoters may have diverged considerably from those of *M. smegmatis*. Our results suggest that the transcription machineries of *M. smegmatis* and *M. bovis* BCG, a species very closely related to *M. tuberculosis*, may be different. In vitro studies with purified RNA polymerases from *M. smegmatis* and *M. tuberculosis* (or BCG) are required to determine whether this is the case.

Cloning and assessment of *M. tuberculosis* gene regulatory sequences. A library of partially *Sau*3AI-digested *M. tuberculosis* DNA was generated in *E. coli*, using vector pJEM13. Nearly 1,200 clones containing recombinant plasmids were obtained, of which 18% were blue on LB–kanamycin–X-Gal plates. Plasmid DNA from 10 colonies was analyzed. The plasmids carried inserts ranging from 0.2 to 1.5 kbp (data not shown). After amplification and pooling of all kanamycin-resistant *E. coli* clones, plasmid DNA was prepared and used to electroporate *M. smegmatis*. Transformants were selected on LB–kanamycin–X-Gal plates, and approximately 12% of the colonies were blue. Sixteen *M. smegmatis* colonies exhibiting different intensities of blue were selected for β-galactosidase assays, in parallel to *M. smegmatis*(pJEM13) as a control (Fig. 3). Most clones expressed only weak β-galactosidase activity (eight β-galactosidase units for clone 3), and β-galactosidase activity was not detected in the *M. smegmatis*-(pJEM13) control. However, clones 1 and 14 were dark blue

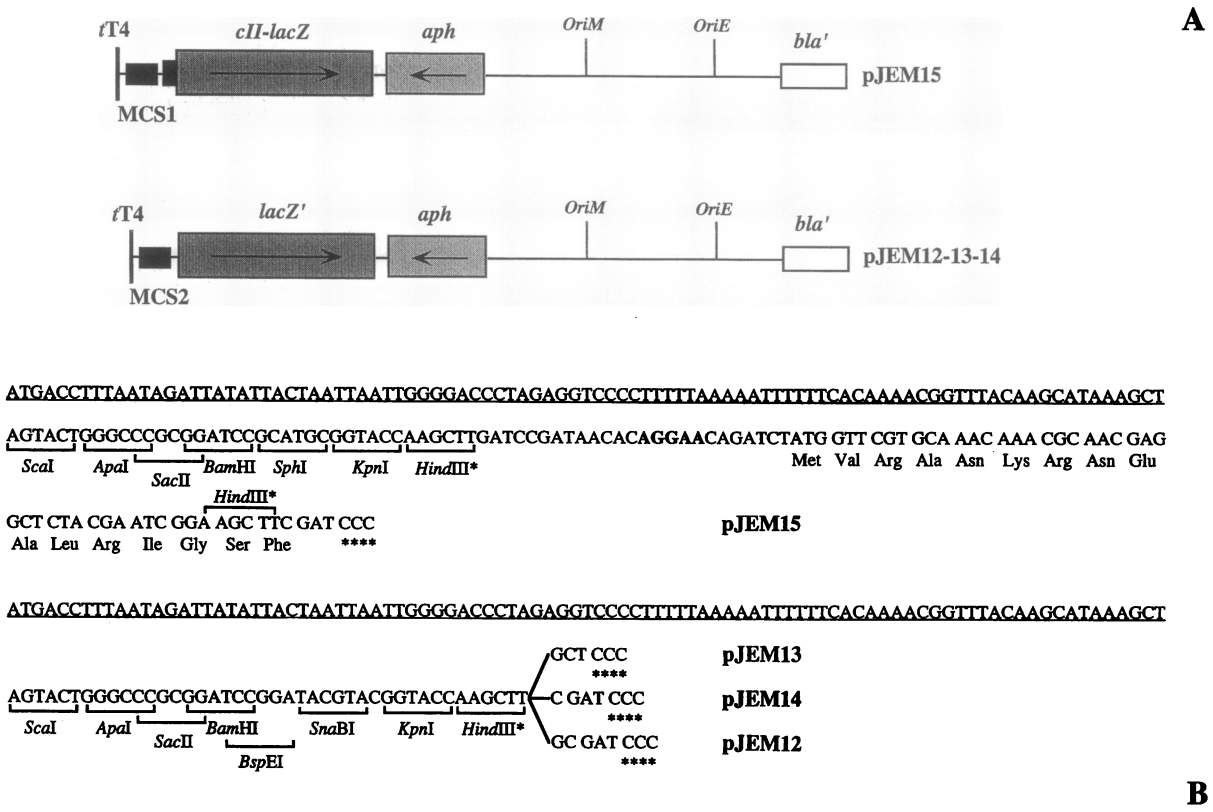

FIG. 2. Structure of plasmids of the pJEM series. (A) Only the relevant genetic elements are indicated. (B) Nucleotide sequences of the regions between primer OJN1 and the eighth codon of *lacZ'* (marked ****). These sequences were verified by DNA sequence determination. The *t*T4 region is underlined, and the sRBS is in boldface. The amino acid sequence of the N terminus of *c*II is given below the DNA sequence. The *Hin*dIII sites are marked with asterisks, as they are not unique. For further descriptions, see the legend to Fig. 1.

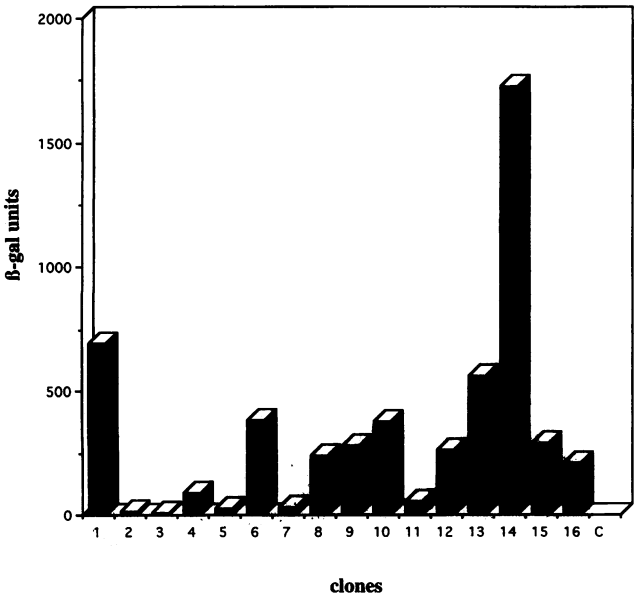

FIG. 3. β-Galactosidase (β-gal) activities of *M. smegmatis* clones containing *M. tuberculosis* gene regulatory sequences. C, the pJEM13 control.

and expressed strong β-galactosidase activity (690 and 1,724 β-galactosidase units, respectively). These clones are very likely to contain strong *M. tuberculosis* promoters; experiments are under way to determine their structures.

The percentage of *M. tuberculosis* DNA fragments with promoter activity in *M. smegmatis* resulting from our experiments (12%) is much higher than the 1 to 2% obtained by Das Gupta and collaborators (6). We suggest that this may be due to a higher sensitivity of the *lacZ* system than the chloramphenicol acetyltransferase system used by these authors. It is possible that selection for resistance to chloramphenicol prevented the isolation of weak promoters.

In conclusion, the family of vectors that we constructed might facilitate the study of gene expression in mycobacteria. A wide range of fragments can readily be cloned in frame to *lacZ'* (gene fusion) or upstream of *c*II-*lacZ* (operon fusion) and be assessed for promoter activity by blue-white screening of mycobacterial transformants on LB–X-Gal plates. Subsequently, the activities of these promoters can be compared (by assaying β-galactosidase activity), their sequences can be determined, and their transcription start sites can be mapped (by primer extension analysis), using the universal primer or related sequences (12) as the primer.

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REFERENCES

1. **Barletta, R. G., D. D. Kim, S. B. Snapper, B. R. Bloom, and W. R. Jacobs.** 1992. Identification of expression signals of the mycobacteriophages Bxb1, L1 and TM4 using the *Escherichia-Mycobacterium* shuttle plasmids pYUB75 and pYUB76 designed to create translational fusions to the *lacZ* gene. J. Gen. Microbiol. **138:**23–30.
2. **Baulard, A., C. Jourdan, A. Mercenier, and C. Locht.** 1992. Rapid mycobacterial plasmid analysis by electroduction between *Mycobacterium* spp. and *Escherichia coli*. Nucleic Acids Res. **20:**4105.
3. **Brown, A., C. E. Hormaeche, R. Dermaco de Hormaeche, M. Winther, G. Dougan, D. J. Maskell, and B. A. D. Stocker.** 1987. An attenuated *aroA Salmonella typhimurium* vaccine elicits humoral and cellular immunity to cloned β-galactosidase in mice. J. Infect. Dis. **155:**86–92.
4. **Casadaban, M. J., J. Chou, and S. N. Cohen.** 1980. In vitro gene fusions that join enzymatically active β-galactosidase segment to amino-terminal fragments of exogenous proteins: *Escherichia coli* plasmid vectors for detection and cloning of translational initiation signals. J. Bacteriol. **143:**971–980.
5. **Clark-Curtiss, J. E., W. R. Jacobs, M. A. Docherty, L. R. Ritchie, and R. Curtiss III.** 1985. Molecular analysis of DNA and construction of genomic libraries of *Mycobacterium leprae*. J. Bacteriol. **161:**1093–1102.
6. **Das Gupta, S. K., M. D. Bashyam, and A. K. Tyagi.** 1993. Cloning and assessment of mycobacterial promoters by using a plasmid shuttle vector. J. Bacteriol. **175:**5186–5192.
7. **Garcia-del-Portillo, F., J. W. Foster, M. E. Maguire, and B. Brett Finlay.** 1992. Characterization of the micro-environment of *Salmonella typhimurium*-containing vacuoles within MDCK epithelial cells. Mol. Microbiol. **6:**3289–3297.
8. **Guglielmi, G., A. Duchêne, C. Thompson, and P. Mazodier.** 1993. Transcriptional analysis of two different *Streptomyces albus groEL*-like genes, p. 17–24. *In* R. H. Baltz, G. D. Hegeman, and P. L. Skatrud (ed.), Industrial microorganisms: basic and applied genetics. American Society for Microbiology, Washington, D.C.
9. **Guillot, C., B. Gicquel, and C. Martin.** 1992. Temperature-sensitive mutants of the *Mycobacterium* plasmid pAL5000. FEMS Microbiol. Lett. **98:**181–186.
10. **Guy, B., M. P. Kieny, Y. Rivière, C. Le Peuch, K. Dott, M. Girard, L. Montagnier, and J. P. Lecocq.** 1987. HIV F/3′ *orf* encodes a phosphorylated GTP-binding protein resembling an oncogene product. Nature (London) **330:**266–269.
11. **Hatfull, G. H.** 1993. Genetic transformation of mycobacteria. TIM **1:**310–314.
12. **Kieser, T., M. T. Moss, J. W. Dale, and D. A. Hopwood.** 1986. Cloning and expression of *Mycobacterium bovis* BCG DNA in "*Streptomyces lividans*." J. Bacteriol. **168:**72–80.
13. **Martín, C., J. Timm, J. Rauzier, R. Gómez-Lus, J. Davies, and B. Gicquel.** 1990. Transposition of an antibiotic resistance element in mycobacteria. Nature (London) **345:**739–743.
14. **Messing, J.** 1983. New M13 vectors for cloning. Methods in enzymology, vol. 204. 20–78.
15. **Miller, J. H.** 1991. Bacterial genetic systems. Methods Enzymol.
16. **Minton, N. P.** 1984. Improved plasmid vectors for the isolation of translational *lac* gene fusions. Gene **31:**269–273.
17. **Murray, A., N. Winter, M. Lagranderie, D. F. Hill, J. Rauzier, J. Timm, C. Leclerc, K. M. Moriarty, M. Gheorghiu, and B. Gicquel.** 1992. Expression of *Escherichia coli* β-galactosidase in *Mycobacterium bovis* BCG using an expression system isolated from *Mycobacterium paratuberculosis* which induced humoral and cellular immune responses. Mol. Microbiol. **6:**3331–3342.
18. **Pardee, A. P., F. Jacob, and J. Monod.** 1959. The genetic control and cytoplasmic expression of "inductibility" in the synthesis of β-galactosidase and tryptophanase induction in *E. coli*. J. Mol. Biol. **1:**656–664.
19. **Prentki, P., and H. M. Krisch.** 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene **29:**303–313.
20. **Ranes, M. G., J. Rauzier, M. Lagranderie, M. Gheorghiu, and B. Gicquel.** 1990. Functional analysis of pAL5000, a plasmid from *Mycobacterium fortuitum*: construction of a "mini" mycobacterium-*Escherichia coli* shuttle vector. J. Bacteriol. **172:**2793–2797.
21. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
22. **Sirakova, T. D., S. S. Bardarov, J. I. Krialov, and K. I. Markov.** 1989. Molecular cloning of mycobacterial promoters in *Escherichia coli*. FEMS Microbiol. Lett. **59:**153–156.
23. **Snapper, S. B., R. E. Melton, S. Mustapha, T. Kieser, and W. R. Jacobs.** 1990. Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. Mol. Microbiol. **4:**1911–1919.
24. **Timm, J., and C. Martín.** Unpublished data.
25. **Timm, J., M. G. Perilli, C. Duez, J. Trias, G. Orefici, L. Fattorini, G. Amicosante, A. Oratore, B. Joris, J. M. Frère, A. P. Pugsley, and B. Gicquel.** 1994. Transcription and expression analysis, using *lacZ* and *phoA* gene fusions, of *Mycobacterium fortuitum* β-lactamase genes cloned from a natural isolate and a high-level β-lactamase producer. Mol. Microbiol. **12:**491–504.
26. **Ullmann, A.** 1984. One-step purification of hybrid proteins which have β-galactosidase activity. Gene **29:**27–31.
27. **Winter, N., M. Lagranderie, J. Rauzier, J. Timm, C. Leclerc, B. Guy, M. P. Kieny, M. Gheorghiu, and B. Gicquel.** 1991. Expression of heterologous genes in *Mycobacterium bovis* BCG: induction of a cellular response against HIV-1Nef protein. Gene **109:**47–54.