Cloning and Assessment of Mycobacterial Promoters by Using a Plasmid Shuttle Vector

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We have constructed a promoter selection vector for mycobacteria to analyze the sequences involved in mycobacterial transcriptional regulation. The vector pSD7 contains extrachromosomal origins of replication from Escherichia coli as well as from Mycobacterium fortuitum and a kanamycin resistance gene for positive selection in mycobacteria. The promoterless chloramphenicol acetyltransferase (CAT) reporter gene has been used to detect mycobacterial promoter elements in a homologous environment and to quantify their relative strengths. Using pSD7, we have isolated 125 promoter clones from the slowly growing pathogen Mycobacterium tuberculosis H37Rv and 350 clones from the fast-growing saprophyte Mycobacterium smegmatis. The promoters exhibited a wide range of strengths, as indicated by their corresponding CAT reporter activities (5 to 2,500 nmol/min/mg of protein). However, while most of the M. smegmatis promoters supported relatively higher CAT activities ranging from 100 to 2,500 nmol/min/mg of protein, a majority of those from M. tuberculosis supported CAT activities ranging from 5 to only about 100 nmol/min/mg of protein. Our results indicate that stronger promoters occur less frequently in the case of M. tuberculosis compared with M. smegmatis. To assess the extent of divergence of mycobacterial promoters vis-à-vis those of E. coli, the CAT activities supported by the promoters in E. coli were measured and compared with their corresponding activities in mycobacteria. Most of the mycobacterial promoter elements functioned poorly in E. coli. The homologous selection system that we have developed has thus enabled the identification of mycobacterial promoters that apparently function optimally only in a native environment.

Mycobacterial diseases, primarily tuberculosis and leprosy, continue to be a major health problem the world over. The situation has been aggravated by the potentially catastrophic impact of AIDS on the spread of tuberculosis (4) and the emergence of drug-resistant strains of mycobacteria (7). The molecular genetics of mycobacteria is poorly understood; this can be attributed to various reasons including their slow growth rates, poor transformation efficiencies, and lack of suitable plasmid vectors. However, the isolation of Mycobacterium smegmatis mutants that could be transformed at a high frequency (27) and the identification and characterization of an autonomously replicating plasmid, pALS000, from Mycobacterium fortuitum (18, 22, 26, 27) have alleviated some of these constraints. The recent past has witnessed an increasing use of recombinant DNA techniques in investigating the molecular biology of mycobacteria, and this may provide better methods for an effective control of mycobacterial diseases (10).

Escherichia coli and Streptomyces lividans have been used to study the expression of mycobacterial genes (15, 17, 19, 30, 32, 33). The efficiency of these heterologous expression systems is, however, variable and does not permit expression of a majority of mycobacterial genes. In addition, host physiological signals (low pH, oxidative stress inside macrophages, iron deficiency, etc.) encountered by mycobacteria during infection can influence the expression of mycobacterial genes, as is known for other pathogenic bacteria (11, 28). To understand the genetic responses elicited by mycobacteria during host-pathogen interactions, it would be important to study the regulation of mycobacterial gene expression in homologous systems that would respond faithfully to various physiological constraints imposed by the host environment. Although some vectors have been constructed recently for the expression of genes in mycobacteria (1, 2, 13, 14, 26, 29), the repertoire of such systems is limited. A major obstacle in the development of such vectors has been the lack of information on mycobacterial transcription signals.

Compared with other bacterial systems, mycobacteria have a low transcription rate which is also manifested in a very low content of RNA per unit of DNA in these organisms (12). Harshney and Ramakrishnan have reported that the rate of incorporation of ribonucleotides into RNA in Mycobacterium tuberculosis H37Rv is about 10-fold lower than that in E. coli (12). The difference in total rate of RNA synthesis is, however, even more pronounced, implying that the rate of initiation of transcription in mycobacteria is probably extremely low (12). Studies on mycobacterial RNA polymerase show that these differences cannot be entirely attributed to an inherent low activity of the enzyme (12). The answer presumably lies in the structure of mycobacterial promoter regions. Upstream regions of mycobacterial genes have been shown to have a higher G + C content than their E. coli counterparts, indicating that mycobacterial promoters might have a high G + C content (8). Attempts have been made in the past to isolate mycobacterial promoters either in E. coli (25) or in S. lividans (17). However, these heterologous systems are unlikely to lead to selection of promoters which function exclusively in mycobacteria. A homologous selection system is essential for the isolation of such promoters. A detailed study of mycobacterial promoters may not only shed light on the extent of divergence of the mycobacterial transcriptional machinery from those of other bacteria but also lead to a better understanding of the genetic

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basis for the observed differences in the growth rates of various mycobacteria.

We report here the construction of a vector, pSD7, which permits study of mycobacterial promoters in their native environments. This vector was used to isolate promoters from the slowly growing pathogen *M. tuberculosis* H37Rv and the fast-growing saprophyte *M. smegmatis* and to assess the strengths of these transcriptional signals. The studies also suggest that most of the mycobacterial promoters function poorly in *E. coli*, indicating thereby that these promoters might be structurally different from their well-studied *E. coli* counterparts.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** *M. smegmatis* LR222 (a high-frequency transforming strain), a generous gift from J. T. Crawford, Centers for Disease Control and Prevention, Atlanta, Ga., was used as the host for isolation of mycobacterial promoters. All transformations in *E. coli* were performed with strain DH5α. The plasmid pKK223-8 (3) was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Plasmid pYUB12 (27) was a gift from T. Kieser, John Innes Institute, Norwich, United Kingdom. Genomic DNA of *M. smegmatis* and *M. tuberculosis* H37Rv were kindly provided by J. S. Tyagi, All India Institute of Medical Sciences, New Delhi, India.

**Enzymes and Chemicals.** Restriction enzymes, T4 DNA ligase, and calf intestinal alkaline phosphatase were purchased from New England Biolabs, Inc., Beverly, Mass. (14C)chloramphenicol (specific activity, 50 mCi/mmol) was purchased from Amersham International plc., Amersham, United Kingdom, and acetyl coenzyme A was from Sigma Chemical Co., St. Louis, Mo.

**Bacterial culture methods and transformation.** *M. smegmatis* was grown in Luria-Bertani (LB) broth supplemented with glycerol (0.5%) and Tween 80 (0.2%) or on LB agar supplemented with glycerol (0.5%). *M. smegmatis* LR222 cells were grown to an A₆₀₀ of 1.0, pelleted by centrifugation at 4,500 × g for 10 min, washed once with 10% glycerol, and resuspended in 10% glycerol (1/50th of the original volume).

The DNA sample was mixed with 20 µl of cell suspension, and the mixture was pulsed at a field strength of 8 to 10 kV/cm with a cell porator (Bethesda Research Laboratories, Gaithersburg, Md.). Cells were recovered in 1 ml of SOC (20) at 37°C with vigorous shaking for 8 h and then plated on LB agar containing kanamycin (25 µg/ml) either alone or in combination with chloramphenicol (20 µg/ml) and incubated at 37°C for 3 days. *E. coli* DH5α cells were grown in LB broth or on LB agar and were transformed by the standard CaCl₂ method (20). Recombinant plasmids were isolated from *M. smegmatis* by a minor modification of the alkaline sodium dodecyl sulfate (SDS) lysis procedure (20).

**Construction of pSD7.** A 2.5-kb EcoRV-HpaI fragment (containing sequences required for autonomous replication in mycobacteria) from pYUB12 was cloned into the SmaI site of pKK232-8, resulting in pSD1 (Fig. 1). A chemically synthesized rho-independent trp terminator (TER3) (23) was then cloned downstream of the mycobacterial fragment, generating pSD2. The nucleotide sequence at the two ends of the synthetic terminator was such that upon cloning, only the BamHI site downstream was generated. This BamHI site was used for cloning fragments for promoter selection (Fig. 1).

An NdeI-DraI fragment of pSD2, containing the ColE1 origin of replication and the ampicillin resistance gene, was replaced by a fragment containing the p15A origin of replicator and the kanamycin resistance gene derived from pSD4, an *E. coli*-mycobacterium shuttle vector constructed in our laboratory for the expression of genes in mycobacteria (8a). The resulting plasmid, pSD7 (Fig. 1), was used for the selection of promoters in mycobacteria.

**Isolation of mycobacterial promoters with pSD7.** Genomic DNA from either *M. tuberculosis* H37Rv or *M. smegmatis* was digested with Sau3AI and fractionated by agarose gel electrophoresis, and fragments from 50 to 800 bp were electroeluted and purified. Approximately 75 ng of purified genomic DNA fragments was ligated to 300 ng of dephosphorylated, BamHI-cleaved pSD7 in a final volume of 10 µl. To estimate ligation efficiencies, a part of the ligation mixture (3 µl) was used to transform *E. coli* and plated on kanamycin (25 µg/ml). The number of colonies obtained was compared with that obtained in a control ligation experiment using 300 ng of dephosphorylated vector. The rest of the ligation mixture (7 µl) was then electroeluted into *M. smegmatis*. The transformed cells were plated on LB agar containing either kanamycin alone (25 µg/ml) or in combination with chloramphenicol (20 µg/ml). The colonies that appeared on kanamycin-chloramphenicol were purified on kanamycin-chloramphenicol plates and subsequently inoculated into kanamycin (25 µg/ml)-containing medium for either plasmid isolation or for chloramphenicol-acetyltransferase (CAT) assay. The frequency of promoter isolation was expressed as the number of kanamycin-chloramphenicol-resistant clones per 100 clones resistant to kanamycin alone.

**Estimation of mycobacterial promoter strength by CAT assay.** For determining the CAT activity, *E. coli* transformants were grown in LB broth containing kanamycin (25 µg/ml). *M. smegmatis* transformants were grown in LB broth containing kanamycin (25 µg/ml), 0.2% Tween 80, and 0.5% glycerol. The CAT-specific activity in cell extracts was determined by the method of Gorman et al. (9). Cells harvested from 3-ml cultures were lysed by sonication in 1 ml of 0.25 M Tris-HCl, pH 7.4, and centrifuged at 8,000 × g for 10 min at 4°C. Assays were performed by incubating 2 µl of extract (unless otherwise stated) with 10 nmol of (14C)chloramphenicol in the presence of 1 mM acetyl coenzyme A in a final volume of 100 µl containing 0.25 M Tris-HCl, pH 7.4. Reactions were carried out for 30 min, after which (14C)chloramphenicol and its acetylated derivatives were separated by thin-layer chromatography as described by Gorman et al. (9). In the cases in which conversion of (14C)chloramphenicol to its acetylated derivatives exceeded the linear range, suitable dilutions of the extracts were used for the assay. CAT specific activity was expressed as nanomoles of chloramphenicol converted into its acetylated derivatives per minute per milligram of protein.

**RESULTS**

**Construction of pSD7.** The vector pSD7 (Fig. 1) consists of the CAT reporter cassette from pKK232-8, the origin of replication from pAL5000, and the kanamycin resistance gene along with the p15A origin of replication derived from pACYC177 (5). The replacement of the ampicillin resistance gene in pSD2 with the kanamycin resistance gene (as in pSD7) was necessary because ampicillin is an unselectable marker in mycobacteria (16). Screening for promoters in the presence of chloramphenicol (20 µg/ml) is based on the activation of the silent CAT gene in pSD7, resulting in resistance to chloramphenicol. Under the experimental conditions used, *M. smegmatis* LR222 showed a reversion
frequency of $10^{-6}$ against chloramphenicol (data not shown). To prevent the growth of any revertants, kanamycin was used along with chloramphenicol.

*M. smegmatis* cells harboring pSD7 exhibited a CAT activity of only 0.9 nmol/min/mg of protein (Table 1) and were sensitive to very low levels of chloramphenicol (<10 μg/ml). The vector pSD7 was used to select promoter clones in mycobacteria by utilizing a selection medium containing kanamycin (25 μg/ml) in addition to chloramphenicol (20 μg/ml).

**Isolation and assessment of mycobacterial promoters.** In order to screen for mycobacterial promoters, ligations and transformations were carried out as mentioned in Materials and Methods. These ligation reactions yielded libraries that were >95% represented by recombinant molecules. The frequency of true promoter clones among these recombi-
nents was 10 to 20% in the case of *M. smegmatis* and 1 to 2% in the case of *M. tuberculosis*. Control transformations with the vector alone gave no transformants when selected on kanamycin plus chloramphenicol. A total of 350 promoter clones from *M. smegmatis* and 125 promoter clones from *M. tuberculosis* H37Rv were isolated. All the promoter plasmids carried inserts ranging from 50 to 800 bp. The strength of the cloned mycobacterial promoters was determined by measurement of CAT activity in cell extracts. A representative CAT assay autoradiogram of individual promoter clones is shown in Fig. 2. A comparison of promoter strengths derived from the two mycobacterial species is depicted in Fig. 3. The vector pSD7 enabled the selection of promoters that varied in strength by at least 2 orders of magnitude; the promoter elements supported CAT activities ranging from about 5.0 nmol/min/mg of protein (S15, T7, T9, and T16) to more than 2,000 nmol/min/mg of protein (S3, S5, T6, and T10). When several promoter clones having different CAT activities, including those having low activities (5 to 10 nmol/min/mg of protein) were used to retransform *M. smegmatis*, chloramphenicol-resistant colonies were observed in all cases. Also the CAT activity in the transformants remained unchanged, indicating that the promoter clones selected were true ones. The assessment of promoters of *M. smegmatis* and *M. tuberculosis* revealed interesting differences in their activities (Fig. 3). Most of the promoters from *M. tuberculosis* supported CAT activities ranging from 5 to 100 nmol/min/mg of protein. On the other hand, the majority of *M. smegmatis* promoters supported CAT activities higher than 500 nmol/min/mg of protein. Only a few promoters of *M. tuberculosis* H37Rv (T3, T6, T10, and T14) supported CAT activities comparable to those of *M. smegmatis*.

**Strength of mycobacterial promoters in E. coli.** To gain an insight into the divergence of mycobacterial promoters vis-à-vis those of *E. coli*, we analyzed the activity of mycobacterial promoters in *E. coli* DH5α. Plasmids isolated from 100 *M. smegmatis* DNA-specific recombinant clones and an equal number of *M. tuberculosis* DNA-specific recombinant clones were individually used to transform *E. coli* and selected on medium containing kanamycin (25 μg/ml) as well as on medium containing chloramphenicol (20 μg/ml) separately. While all the promoter plasmids of *M. smegmatis* and *M. tuberculosis* conferred kanamycin resistance on *E. coli*, only 12 plasmids containing *M. smegmatis* DNA were found to confer chloramphenicol resistance on *E. coli*. Furthermore, none of the promoter plasmids derived from *M. tuberculosis* conferred chloramphenicol resistance on *E. coli*. This implied that a majority of mycobacterial promoters, particularly those of *M. tuberculosis*, did not function optimally in *E. coli*. The CAT activities exhibited by individual *M. smegmatis* and *M. tuberculosis* promoter plasmids in kanamycin-resistant *E. coli* transformants was determined and compared with their corresponding activities in mycobacteria. The results are presented in Fig. 4 and 5. Except for one *M. smegmatis* promoter plasmid (S64) which exhibited comparable CAT activity in *E. coli* and mycobacteria, all of the promoters exhibited low activities in *E. coli* (Fig. 4). The *M. smegmatis* promoters that conferred chloramphenicol resistance on *E. coli* (S13, S16, S30, S73, S86, and S150) supported CAT activities in the range of 100 to 350 nmol/min/mg of protein in *E. coli*, but these were 2- to 20-fold lower than the corresponding values in mycobacteria (Fig. 4). The differences in the activities of these promoters in mycobacteria and *E. coli* were particularly striking in the cases of S3, S5, and S19 (Fig. 4). All the *M. tuberculosis* promoters supported poor CAT activities in *E. coli* (Fig. 5), substantiating the fact that none of these promoter plasmids could confer chloramphenicol resistance on *E. coli*. Some of the *M. tuberculosis* promoters (T3, T6, T10, and T14 (Fig. 5)) exhibited differences of several hundredfold in their activities in mycobacteria and *E. coli*.

**DISCUSSION**

The present study constitutes a systematic approach to isolate and assess mycobacterial transcriptional signals by a screening strategy that selects for promoters in a homologous environment. A promoter-probe vector for mycobacteria which utilizes the CAT reporter gene (9) for both detection and quantification of the strength of the promoter elements has been developed. The activation of CAT reporter gene by mycobacterial promoter-containing DNA fragments cloned upstream of it facilitates the rapid selection of promoter-containing clones by using a selection medium containing chloramphenicol. The frequency of reversion against chloramphenicol obtained in the present study was 10⁻⁶, and even the revertants occurring at this frequency will not grow because of the simultaneous use of kanamycin and chloramphenicol in our selection procedure. Considering even the minimum frequency of promoter isolation (10⁻⁷, as in the case of *M. tuberculosis*), one would expect 10⁶ true promoter clones and 1 false clone out of a library of 10⁹ recombinants. The reasons for a 100-fold-lower frequency of reversion in our study compared with that re-

<table>
<thead>
<tr>
<th>Host and plasmid</th>
<th>Sp act (nmol/min/mg of protein)</th>
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<tbody>
<tr>
<td>E. coli pKK232-8</td>
<td>0.26</td>
</tr>
<tr>
<td>pSD1</td>
<td>33.25</td>
</tr>
<tr>
<td>pSD2</td>
<td>0.60</td>
</tr>
<tr>
<td>pSD7</td>
<td>0.30</td>
</tr>
<tr>
<td>M. smegmatis (pSD7)</td>
<td>0.90</td>
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* E. coli DH5α and *M. smegmatis* LR22 were transformed by using the various vector constructs and selected on kanamycin (25 μg/ml).

* CAT specific activity was determined as described in Materials and Methods.
reported by Snapper et al. (27) are not clear to us at present, but the use of a different strain of *M. smegmatis* and a different medium for culture could be contributory factors. Using the vector pSD7, we have undertaken a study involving a comparative analysis of promoters from the slowly growing pathogen *M. tuberculosis* and the fast-growing saprophyte *M. smegmatis*. The selection frequency of promoters with *M. tuberculosis* DNA was 10 to 20 times

![A](image1.png)

**FIG. 3.** Comparison of strengths of *M. smegmatis* promoters (A) and *M. tuberculosis* H37Rv promoters (B) in *M. smegmatis*. The y axis has been broken from 1,200 to 1,500 in both panels. Promoter strengths were determined by CAT assays performed as described in Materials and Methods. The CAT specific activity is expressed as nanomoles per minute per milligram of protein.

![B](image2.png)

**FIG. 4.** Comparative activities of *M. smegmatis* promoters in *M. smegmatis* (solid bars) and in *E. coli* (diagonally cross-hatched bars). The CAT specific activity was determined as described in Materials and Methods and is expressed as nanomoles per minute per milligram of protein.

![C](image3.png)

**FIG. 5.** Comparative activities of *M. tuberculosis* promoters in *M. smegmatis* (solid bars) and in *E. coli* (diagonally cross-hatched bars). The y axis has been broken at 150. The CAT specific activity was determined as described in Materials and Methods and is expressed as nanomoles per minute per milligram of protein.
lower than that obtained with *M. smegmatis* DNA. A higher selection frequency probably could be achieved by lowering the antibiotic dose. However, the results in that case would not be completely free from ambiguity because of a low signal-to-noise ratio. The selection procedure described here was found to be stable. When *M. smegmatis* was retransformed with the promoter plasmids and transformants from kanamycin-chloramphenicol plates were assessed for activity, no significant change was observed, indicating against the possibility of any upmutations being involved in the process of promoter selection. The results of the CAT assay (Fig. 3) indicate that stronger promoters occur less frequently in the case of *M. tuberculosis* compared with in *M. smegmatis*. This is consistent with the lower frequency of promoter isolation in the case of *M. tuberculosis* compared with that of *M. smegmatis*. It is conceivable that the promoters of *M. tuberculosis* might have diverged from those of *M. smegmatis* to such an extent that they do not function optimally in *M. smegmatis*. Testing the activity of the *M. tuberculosis* H37Rv promoters in a more homologous system, such as *M. tuberculosis* H37Ra (an avirulent derivative of *M. tuberculosis* H37Rv), might provide definite answers. However, considering a lower rate of transcription (12), slower growth rate, and lower content of RNA per unit of DNA in the case of *M. tuberculosis* compared with those of *M. smegmatis*, it seems more likely that a majority of promoters of *M. tuberculosis* H37Rv might be inherently weaker than those of *M. smegmatis*.

A major advantage of the vector pSD7 is that the promoters detected in mycobacteria could be directly assessed in *E. coli* without an additional subcloning step. Such a direct comparison provides an opportunity to examine the extent of divergence of mycobacterial promoters from their well-characterized *E. coli* counterparts. Only about 12% of *M. smegmatis* promoters were expressed in *E. coli*, as indicated by their ability to confer chloramphenicol resistance. Moreover, with the exception of the promoter plasmid S64, all *M. smegmatis* promoters supported relatively lower CAT activities in *E. coli* than in mycobacteria (Fig. 4). The difference varied from a marginal 2-fold (S30) to a substantial 100-fold (S5). The functioning of *M. tuberculosis* promoters at a similar level in *E. coli* was even more striking, as none of the promoter plasmids conferred chloramphenicol resistance on *E. coli* and exhibited little or no CAT activity in *E. coli* (Fig. 5). In fact, some promoters, viz., T3, T6, T10, and T14 (Fig. 5), which supported substantially high CAT activity when assayed in mycobacteria evinced virtually no response in *E. coli*. The poor functioning of mycobacterial promoters in *E. coli* compared with that in *M. smegmatis* was not due to a lower copy number of pSD7 in the former; the presence of the p15A-derived origin of replication in pSD7 maintains a copy number of 15 to 20 in *E. coli* (5) compared with the origin of replication of pAL5000, which provides only about 3 copies in mycobacterium (22). Our data suggest that the number of mycobacterial genes whose promoter signals are recognized in *E. coli* might not be substantial (Fig. 4 and 5) and provide a plausible explanation as to why the attempts to express mycobacterial genes in heterologous systems such as *E. coli* under the control of mycobacterial transcriptional signals have met with only limited success (6, 15). Significantly, the few mycobacterial genes that have been expressed in *E. coli* apparently from their own promoters belong to the heat shock protein family, members of which are conserved across evolution (21, 24, 30, 31). However, it is not clear whether the expression of heat shock genes or other conserved genes of mycobacteria in *E. coli* reflects their optimal expression in mycobacteria. In this context it is interesting to note that when the rRNA gene promoter of *M. tuberculosis* H37Rv was cloned in pSD7, it supported an activity of 17,159 nmol/min/mg of protein in *M. smegmatis* and exhibited an activity of only 1,593 nmol/min/mg of protein in *E. coli* (31a).

In conclusion, we have isolated several promoters of *M. tuberculosis* H37Rv and *M. smegmatis* by using a promoter selection vector that was constructed for the identification of promoter elements in a homologous environment. The promoters exhibited a wide range of strengths in mycobacteria, functioned poorly in *E. coli*, and appeared to be dependent on the availability of a homologous environment for optimal expression. The identification of promoter-containing clones should pave the way for the development of expression vectors for mycobacteria. In addition, sequence analysis, promoter mapping experiments, and studies involving DNA-protein interactions should shed light on the structure of regulatory elements involved in efficient and accurate transcription in mycobacteria.

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