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Cloning and Expression of *Mycobacterium bovis* BCG DNA in "*Streptomyces lividans*"

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The ability of "*Streptomyces lividans*" to use the expression signals of genes from *Mycobacterium bovis* BCG was tested in vivo by using gene fusions. Random DNA fragments from *M. bovis* BCG were inserted into promoter-probe plasmids in *Escherichia coli* and in "*S. lividans*." Comparison with promoter activity detected with random DNA fragments from the respective hosts suggested that "*S. lividans*" efficiently utilizes a high proportion of mycobacterial promoters, whereas a smaller fraction are expressed, and expressed more weakly, in *E. coli*. *M. bovis* BCG DNA fragments were also inserted into the specially constructed translational fusion vector (pIJ688) in "*S. lividans*." pIJ688 contains the kanamycin phosphotransferase gene (*neo*) from transposon Tn*5*, truncated at its amino terminus, as the indicator. The results suggested that "*S. lividans*" uses *M. bovis* BCG translational signals almost as efficiently as its own signals. Moreover, several hybrid proteins with an *M. bovis* BCG-derived amino terminus seemed to be reasonably stable in "*S. lividans*." These experiments indicate that "*S. lividans*" may be a suitable host for the expression of *Mycobacterium leprae* and *Mycobacterium tuberculosis* genes from their own signals. This is a precondition for the expression of entire biosynthetic pathways, which could be valuable in the production of diagnostic and therapeutic agents. The vectors may also have wider applications for the analysis of gene expression in *Streptomyces*.

Tuberculosis and leprosy are still among the world's major infectious diseases. They are caused by the slow-growing *Mycobacterium tuberculosis* and *Mycobacterium leprae*, both of which grow intracellularly in the host (obligately in the case of *M. leprae*), making chemotherapy difficult. The slow growth rates of both pathogens (doubling times in vivo of 15 to 20 h and 12 to 20 days, respectively) and the failure of *M. leprae* to grow in vitro have constrained the study of these bacteria. Gene cloning provides a way of producing mycobacterial proteins in more convenient organisms. The availability of such gene products should help in the refinement of diagnostic agents and possibly the development of vaccines, as well as in screening for chemotherapeutic agents targeted on specific mycobacterial enzymes. The latter becomes increasingly important with the emergence of resistant strains.

Recently, considerable progress has been made in the expression of mycobacterial genes in *Escherichia coli*. Experiments in which *M. leprae* DNA was cloned in *E. coli* cosmid vectors suggested that mycobacterial genes could, at best, be expressed only weakly from their own signals in this host (8), a conclusion reinforced by cloning *M. bovis* BCG DNA in lambda EMBL 3 (28). By the use of expression vectors, clones were isolated which complemented *E. coli* auxotrophic mutations (8) or which produced polypeptides reacting with monoclonal antibodies specific for *M. leprae* and *M. tuberculosis* (28, 34, 35). A particular *Mycobacterium fortuitum* plasmid gene was shown to be expressed from its own promoter (21).

The use of an alternative host which would express mycobacterial genes without requiring an expression vector not only would accelerate the search for further mycobacterial genes, but might also make it possible to obtain expression of multigene pathways. These pathways would include that responsible for the biosynthesis of the *M. leprae*-specific phenolic glycolipid which is an immunodominant antigen (15, 33). This objective should be aided by the general tendency of genes for biosynthetic pathways to be clustered in bacterial genomes. Nonpathogenic relatives of pathogenic *Mycobacterium* strains are the most likely hosts to express *M. tuberculosis* or *M. leprae* genes, but cloning procedures for mycobacteria have not yet been developed. Streptomycetes are nonpathogenic gram-positive bacteria which belong, in common with the mycobacteria, to the order *Actinomycetales*, even though the G+C content of *Streptomyces* spp. DNA (about 73% [9]) is higher than that of the mycobacteria (56 to 65% [8]). Efficient cloning and in vivo genetic procedures are available for "*Streptomyces lividans*" (13), which is a convenient, nonrestricting host. Moreover, existing evidence suggests that streptomycetes are quite versatile in their ability to recognize heterologous procaryotic promoters (2, 16). (*Streptomyces* promoters are themselves heterogeneous in sequence [12], and multiple forms of RNA polymerase are at least partly responsible for the transcription of different promoter classes [32].)

To investigate the general feasibility of cloning and expressing *Mycobacterium* genes in "*S. lividans*," we have explored the ability of transcription and translation signals in DNA from the nonpathogenic *M. bovis* BCG strain to activate indicator genes in suitable plasmid vectors. The level of in vivo expression of the indicator gene was used to survey the strength of the cloned signals, and the result was assessed by comparing the level of expression achieved with random heterologous (in our case BCG) or homologous ("*S. lividans*") DNA fragments. A feature of this approach is that, with a single assay, the efficiency of a random sample of all promoters and translational signals can be examined in vivo without prior knowledge of gene functions. The results are of course influenced by other factors, notably the stability of the hybrid mRNAs and, in translational fusions, of the hybrid proteins. However, since we are primarily interested in the production of mycobacterial proteins in "*S. lividans*"

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rather than in a precise assessment of the contributions of different factors to overall gene expression, this could be an advantage. We have used high-copy-number *Streptomyces* plasmid vectors which might be expected to lead to increased production of proteins from some of the cloned genes through gene amplification. The *neo* gene from the *E. coli* transposon Tn*5* was the indicator gene. This gene codes for an aminoglycoside phosphotransferase (NPT II) that confers resistance to kanamycin (or neomycin) on "*S. lividans*." The level of resistance varies widely, depending on the amount of NPT II produced, and can therefore be used as a convenient indicator of the level of gene expression.

MATERIALS AND METHODS

Bacterial strains and plasmids. "*S. lividans*" 66 strain TK64 (*str-6 pro-6*, plasmid-free) (14) was the host for transformation with pIJ424 and its derivatives (31a). *E. coli* HB101 (6) was the host for transformation with pKK232-8 (P-L Biochemicals) (7) and its derivatives. *M. bovis* BCG (Glaxo) was obtained from J. Morris (Central Veterinary Laboratory, Weybridge, U.K.).

Culture media, antibiotic selection, and transformation procedures. Techniques for streptomycetes were as described in detail by Hopwood et al. (13). "*S. lividans*" was grown in liquid YEME medium with 34% sucrose and 0.5% glycine for protoplasting and for the preparation of protein extracts. Tryptone soya broth (Oxoid, CM 129) was used to grow mycelium for plasmid isolation. R2YE agar was used for the regeneration of protoplasts and for the preparation of spore suspensions, and MM agar was used for the selection of kanamycin-resistant strains. All media for streptomycetes were supplemented with proline to allow growth of strain TK64 and with thiostrepton when appropriate (50 μg/ml for solid media and 5 μg/ml for liquid media). Transformation of "*S. lividans*" protoplasts was done by the small-scale procedure of Hopwood et al. (13). *E. coli* was grown in L broth or on L agar (27) supplemented with ampicillin (50 μg/ml), kanamycin sulfate (50 μg/ml), and various concentrations of chloramphenicol, as appropriate. Transformation of *E. coli* with plasmid DNA was done by the method of Kushner (20). *M. bovis* BCG was grown in Santon medium, pH 7.0, containing (per liter) 0.5 g of K_2HPO_4, 2 g of citric acid, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of ammonium ferric citrate, 20 g of glycerol, 4 g of asparagine, and 0.2 g of Tween 80 with vigorous shaking at 37°C for 3 weeks.

Manipulation of DNA. Chromosomal DNA from all three species was prepared by procedure 3 of Hopwood et al. (13). Plasmid DNA was prepared by the method of Kieser (18) or, for some of the *E. coli* preparations, by that of Birnboim and Doly (4). Plasmid vectors were purified either by two rounds of cesium chloride-ethidium bromide gradient centrifugation or by electroelution from low-melting-temperature agarose gels (LMP agarose; Bethesda Research Laboratories, catalog no. 5517 UB) after digestion with restriction enzymes and treatment with alkaline phosphatase. The eluted DNA was purified as recommended by Zimmermann and Harrison (36). In vitro manipulation of DNA was done by standard procedures as described in Hopwood et al. (13).

Preparation of cell extracts. Soluble protein extracts from "*S. lividans*" were prepared from mycelium washed twice with the sample buffer of Reiss et al. (25). The cells were broken by sonication in sample buffer, and the lysate was spun for 10 min at 4°C in an Eppendorf centrifuge to remove cell debris. Samples were stored at −70°C.

Polyacrylamide gel electrophoresis. Polyacrylamide (10%) gels were prepared, run, and stained with Coomassie blue by procedure 53 of Silhavy et al. (27). For nondenaturing gels, sodium dodecyl sulfate (SDS) was omitted from all the buffers and the samples were not boiled. About 50 μg of protein was loaded into each gel slot.

Detection of kanamycin phosphotransferase activity in native polyacrylamide gels. The procedure used to detect kanamycin phosphotransferase activity was that of Reiss et al. (25) with the following specifications: 100 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol) was used for each agarose gel (150 by 200 by 1 mm). The P81 phosphocellulose paper was washed three times each for 1 h with 50 mM sodium phosphate buffer, pH 7.4, and twice with cold water before being dried and exposed to X-ray film.

Detection of kanamycin phosphotransferase by Western blotting. Proteins separated on SDS-polyacrylamide gels were transferred electrophoretically to nitrocellulose (Schleicher & Schuell BA85) (29) with 25 mM sodium phosphate buffer, pH 6.5, at room temperature with a Bio-Rad Trans-Blot Cell (ca. 10 V and 0.2 A for 14 h). The filter was washed and reacted sequentially with 1:1,000 diluted rabbit anti-NPT II serum (a kind gift from J. Davies, Biogen S.A., Geneva) and 1:500 diluted goat anti-rabbit immunoglobulin G (IgG) conjugated to alkaline phosphatase (kindly prepared by R. Casey, John Innes Institute), and then stained by the procedure of Blake et al. (5).

RESULTS

Cloning of *M. bovis* BCG DNA fragments into the *Streptomyces* promoter-probe plasmid pIJ424. pIJ424 (31a) carries the replication region of the high-copy-number, wide-host-range *Streptomyces* plasmid pIJ101 (19), the thiostrepton resistance gene (*tsr*) for selection, the promoterless kanamycin resistance determinant (*neo*), and a transcriptional terminator (*ter*) from the *E. coli* phage fd (Fig. 1). Between the unique *Bgl*II cloning site (which is downstream from *ter*) and the ATG start codon for *neo* are an in-frame stop codon (which prevents the formation of in-frame fusions to *neo*) and a ribosome binding site (Shine-Dalgarno sequence). The *neo* gene is activated when a DNA fragment with promoter activity is inserted in the correct orientation into the *Bgl*II site. The level of kanamycin resistance conferred on "*S. lividans*" varies between about 2 and more than 500 μg/ml, depending on the strength of the cloned promoter.

To generate a BCG gene library in "*S. lividans*," 5 μg of BCG DNA was digested with *Bgl*II and ligated to 1 μg of pIJ424 digested with *Bgl*II and dephosphorylated with calf intestinal alkaline phosphatase (CIAP). After transformation of "*S. lividans*" protoplasts, about 150,000 thiostrepton-resistant transformants were selected and tested for growth on kanamycin (5 μg/ml). An estimated 50,000 of these colonies were resistant to kanamycin. Analysis by agarose gel electrophoresis of plasmid DNA from 80 such colonies showed that all clones contained plasmids larger than the vector. Most inserts were between 0.5 and 10 kilobases (kb) in size (average, 5 kb), reflecting the size distribution of the *Bgl*II-cut BCG DNA, and thus probably represented a random sample of BCG DNA. There was no indication of the multiple occurrence of particular DNA fragments. This experiment suggested that BCG DNA contains a large number of promoters active in "*S. lividans*."

The following control experiments confirmed that DNA fragments from BCG had been cloned and were responsible for the kanamycin resistance of the clones.

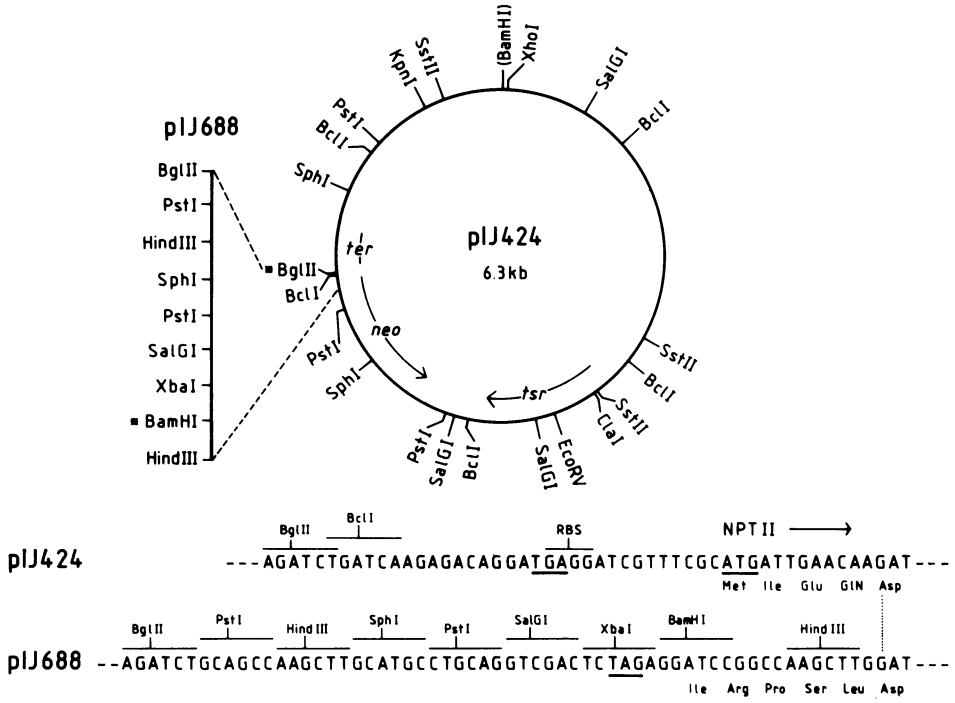


FIG. 1. Restriction maps of the *Streptomyces* promoter-probe plasmid pIJ424 and the translational fusion vector pIJ688. The two plasmids differ at the amino terminus of the *neo* gene, and pIJ688 lacks the *Bam*HI site shown in parentheses. (The derivation of pIJ688 from pIJ424 is explained in Fig. 4.) The sequence at the amino terminus of the *neo* gene in pIJ424 is from Beck et al. (1) and was confirmed in a derivative of pIJ424 by Ward et al. (31a). The sequence of pIJ688 is deduced from the results of Reiss et al. (25) for pKM109-90 and Ward et al. (31a) for pIJ486. *neo*, Kanamycin phosphotransferase gene from Tn*5*; *ter*, transcriptional terminator from phage fd (10); *tsr*, thiostrepton resistance determinant; RBS, ribosome binding site. Stop codons and the ATG start codon for the *neo* gene are underlined. Asterisks indicate the cloning sites.

Plasmid DNA from 12 kanamycin-resistant clones was purified and reintroduced into "*S. lividans*" protoplasts. All transformants were kanamycin resistant, confirming that in each case the resistance was plasmid-borne. The 12 plasmids were digested with *Bgl*II and religated, regenerating the original pIJ424 vector. After introduction into "*S. lividans*," thiostrepton-resistant transformants were recovered and found to be kanamycin sensitive. This confirmed that the kanamycin resistance of the clones was due to the cloned DNA fragments and not a consequence of mutations or rearrangements of the vector plasmid.

To eliminate the possibility that DNA from a chance contaminant had been cloned, DNA was isolated from a second BCG culture. After digestion with *Bgl*II, the sample of DNA used for the cloning experiment and the new sample gave the same characteristic banding patterns on an agarose gel, confirming their identity.

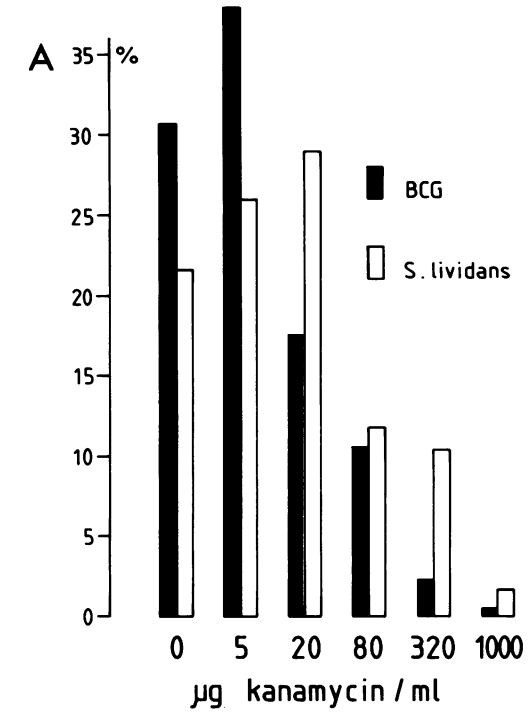
The unlikely possibility that residual chromosomal DNA from "*S. lividans*" in the vector DNA preparation might have been the source of the observed promoter activity was excluded for one clone by using ^{32}P-labeled (nick-translated) plasmid DNA from a highly kanamycin-resistant clone as the probe in a Southern hybridization experiment against *Bgl*II-digested DNA from BCG and "*S. lividans*." The BCG DNA gave a single positive signal in the position corresponding to the size of the cloned fragment, with no hybridization to the "*S. lividans*" DNA.

The above experiments, coupled with the finding that the clones contained plasmids of many different sizes, established that BCG DNA can be cloned with high efficiency in "*S. lividans*" and that many BCG DNA fragments show promoter activity in this host.

When "*S. lividans*" was transformed with undigested pIJ424, fewer than 1% of the thiostrepton-resistant colonies could grow after replication to kanamycin (5 μg/ml). Usually only part of a colony (possibly only a single transferred spore) was kanamycin resistant. The kanamycin-resistant colonies obtained with cloned BCG DNA, on the other hand, were always completely resistant and replicated as small patches. Plasmid DNA from 12 spontaneously kanamycin-resistant mutants was reintroduced into "*S. lividans*." Kanamycin-resistant colonies were recovered, proving that the mutations were plasmid-borne. Two of these plasmids were smaller than pIJ424 and stable, but 10 were larger, of various size, and unstable, regenerating vector-size molecules which no longer conferred kanamycin resistance.

Quantitative comparison of promoter activity in "*S. lividans*" of DNA fragments from BCG or "*S. lividans*." Randomly selected thiostrepton-resistant colonies from the BCG library were tested for their level of kanamycin resistance and for possession of inserted DNA. Fifty percent were found to contain vector plasmids with no detectable insert; all of these were kanamycin sensitive. Of 270 clones which contained BCG DNA, a surprisingly high 69% were resistant to at least 5 μg of kanamycin per ml. Since about 50,000 kanamycin-resistant clones were recovered (see above), an estimated 72,000 BCG clones were obtained in this experiment.

These results can be compared with those obtained with a library of "*S. lividans*" fragments cloned in the same manner. A similar proportion lacked inserts, and again these were all kanamycin sensitive. In this case, of 190 clones containing "*S. lividans*" DNA, 78% were resistant to at least 5 μg of kanamycin per ml. The similarity of this figure to that



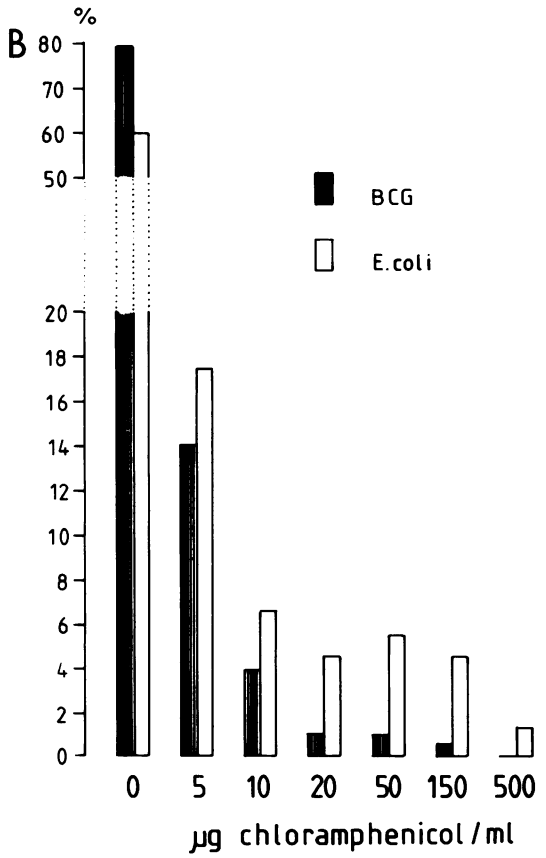


FIG. 2. (A) Kanamycin resistance levels of pIJ424 clones (transcriptional fusions) in "*S. lividans*" TK64 containing *Bgl*II fragments of BCG DNA or "*S. lividans*" DNA. (B) Chloramphenicol resistance levels of pKK232-8 clones (transcriptional fusions) in *E. coli* HB101 containing *Bam*HI fragments of BCG or *E. coli* DNA. The heights of the bars represent the percentages of clones which grew on medium with the indicated concentration of antibiotic but not with the next higher concentration.

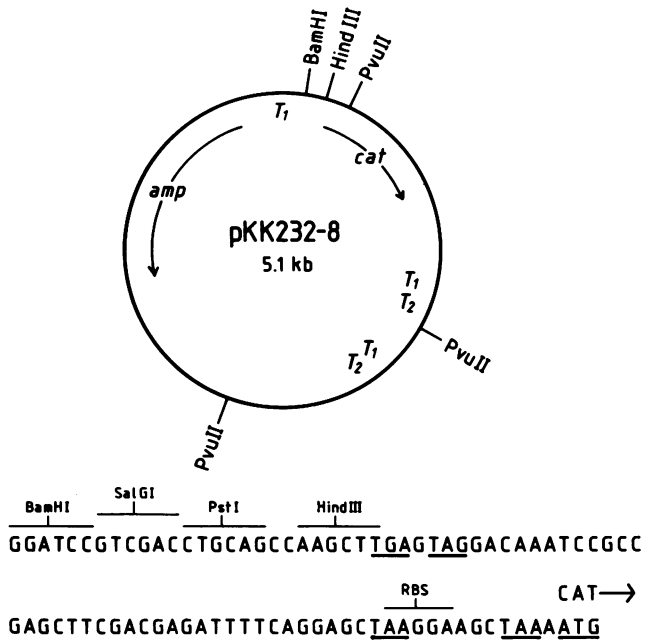


FIG. 3. Simplified restriction map of the *E. coli* promoter-probe plasmid pKK232-8 (7). The main features are the ampicillin resistance determinant (*amp*) for selection and the promoterless chloramphenicol resistance gene (*cat*) which codes for chloramphenicol acetyltransferase (CAT). T_1 and T_2 are transcriptional terminators from the *rrnB* gene. The sequence of the amino-terminal region of the *cat* gene is combined from data published by Brosius (7) and Vieira and Messing (31). The ATG start codon for the *cat* gene and stop codons (present in all three reading frames) are underlined. RBS, Ribosome binding site.

obtained with BCG DNA (69%) suggests that the majority of BCG promoters are recognized in "*S. lividans*." The size distribution of the inserts was approximately the same for both sets of clones.

The relative strengths (in "*S. lividans*") of the BCG and "*S. lividans*" promoters can be assessed by comparing the level of kanamycin resistance of individual clones. Figure 2A shows that fewer of the BCG clones exhibited high-level resistance to kanamycin, indicating that the level of expression from the BCG promoters is on average somewhat lower than that obtained from the homologous "*S. lividans*" DNA fragments.

The isolation of kanamycin-resistant clones at a frequency of more than 50% is remarkable and raises the question whether the detection system was too sensitive, possibly because of the high copy number (ca. 100 per chromosome) of pIJ424, leading to initiation of transcription at DNA sequences which are not used as promoters under normal circumstances. Kanamycin resistance in more than 50% of the clones would be expected, however, if promoters are frequently in a "back to back" arrangement in the genome and if termination of transcription is often incomplete, as is the case in *E. coli* (26).

"*S. lividans*" TK64 containing pIJ424 is slightly more resistant to kanamycin than the plasmid-free strain; the difference can be detected on kanamycin gradient plates (31a). It is not known whether this slight kanamycin resistance is due to incomplete termination at the fd terminator or to initiation of RNA synthesis at a weak promoter downstream of the termination point (R. Gentz, personal communication). Seventy clones sensitive to 5 µg of kanamycin per

ml were tested on gradient plates. Six of these clones were more sensitive to kanamycin than was strain TK64 containing pIJ424 and probably contained transcriptional terminators.

Promoter activity of BCG DNA in *E. coli* HB101. To clarify the expression of mycobacterial genes in *E. coli* and to provide a comparison for the "*S. lividans*" result, a similar experiment was performed with the *E. coli* promoter-probe plasmid pKK232-8 (Fig. 3) (7) and *Bam*HI-generated fragments of DNA from BCG or *E. coli* HB101.

With BCG DNA, 1,043 ampicillin-resistant transformants were obtained; 170 were found to be resistant to at least 5 μg of chloramphenicol per ml, i.e., showed promoter activity, and 873 were sensitive. Plasmids from samples of chloramphenicol-resistant and -sensitive colonies were analyzed; all the resistant colonies contained plasmids with inserts, as did 75% of the sensitive colonies. (The average insert size was 5 kb.) Thus, 21% [100 × 170 ÷ (170 + 873 × 0.75)] of the total clones with inserts showed promoter activity.

With *E. coli* DNA, 115 of 461 ampicillin-resistant transformants were resistant to at least 5 μg of chloramphenicol per ml, and 346 were sensitive. All resistant clones had inserts, as did 50% of the sensitives. (The average insert size was 7.5 kb.) Thus, 40% [100 × 114 ÷ (115 + 346 × 0.5)] of the clones with inserts showed promoter activity. (The fact that this value is much lower than that obtained with homologous DNA in the "*S. lividans*" promoter-probe experiment [78%] may not be biologically significant; it could simply indicate that pKK232-8 is less sensitive in detecting the presence of very weak promoters.)

The relative strengths of the homologous and heterologous promoters in *E. coli* were assessed by testing the levels of chloramphenicol resistance of individual clones (Fig. 2B). A comparison with the "*S. lividans*" results (Fig. 2A) suggests that most BCG promoters compare better with homologous promoters in "*S. lividans*" than in *E. coli*, in which only a small minority of BCG promoters were strongly active.

Construction of pIJ688, a translational fusion vector for streptomycetes. Having established that many BCG promoters are active in "*S. lividans*," we set out to determine whether BCG translational start signals also function in this organism. For this purpose we replaced the promoterless *neo* gene in pIJ424 with the truncated *neo* gene from pKM109-90 (24), which lacks the ribosome binding site and the codons for the first four amino acids, including the ATG start codon (Fig. 1). The construction of pIJ688 is explained in Fig. 4. Kanamycin resistance is expected to occur only when a DNA fragment with a promoter and a translational start signal is fused in the right orientation and reading frame to the amino terminus of the truncated *neo* gene. (Only the unique *Bam*HI site of pIJ688 can be used for making translational gene fusions, because there is an in-frame TAG stop codon in the *Xba*I recognition sequence [Fig. 1].)

Quantitative comparison of kanamycin resistance levels obtained in "*S. lividans*" with DNA fragments from BCG or "*S. lividans*" cloned into the translational fusion vector pIJ688. *Bam*HI-digested BCG and "*S. lividans*" DNA samples were ligated to *Bam*HI-digested and calf intestinal alkaline phosphatase-treated pIJ688 DNA and introduced into "*S. lividans*" by transformation. Samples of the resulting transformants (154 for the experiment with BCG DNA and 149 for that with "*S. lividans*" DNA) were analyzed by gel electrophoresis: 106 and 80 of the colonies, respectively, had detectable inserts. Eight percent (9 of 106) and 13% (10 of 80) of the clones with detectable inserts from BCG and "*S. lividans*" conferred resistance to at least 1.5 μg of kanamycin per ml (pIJ688, unlike the promoter-probe plasmid pIJ424, does not increase the kanamycin resistance of *S. lividans*). Plasmids from eight kanamycin-resistant clones containing BCG DNA inserts were isolated and reintroduced into "*S. lividans*," where again kanamycin resistance was

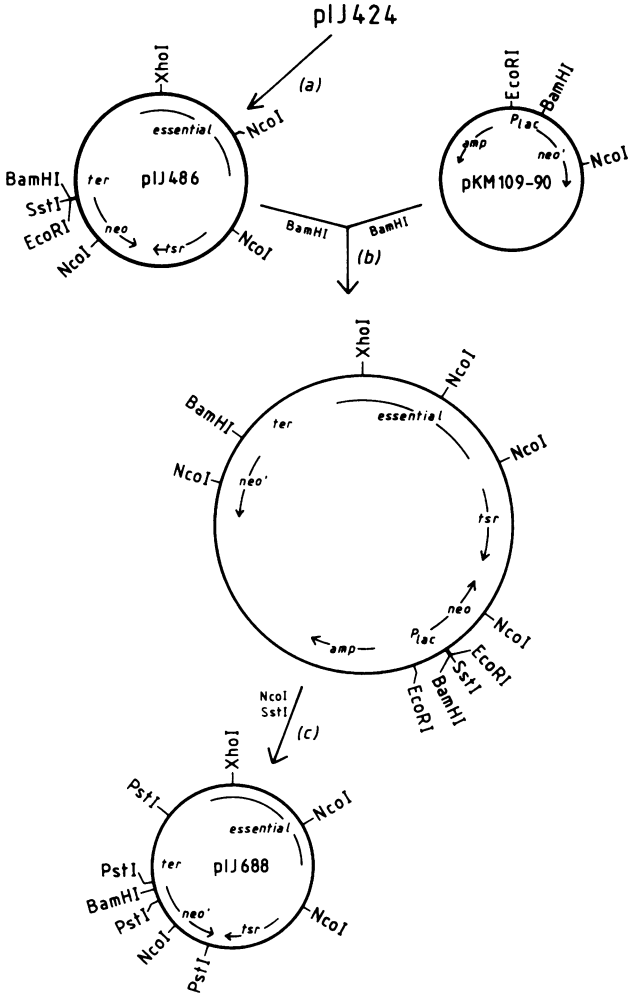


FIG. 4. Construction of the translational fusion vector pIJ688. (a) A synthetic polylinker was inserted into a derivative of pIJ424 lacking the *Bam*HI site by Ward et al. (31a) to give pIJ486. (b) pKM109-90 (24) and pIJ486 were digested with *Bam*HI, ligated and introduced by transformation into *E. coli* ED8767. Kanamycin-resistant colonies had the expected structure. The *neo* gene from pIJ486 is expressed from the *lacUV5* promoter (P_{lac}). (Attempts to make a similar construct by the apparently simpler route of digesting both plasmids with *Bam*HI and *Eco*RI failed repeatedly.) (c) Digestion with *Nco*I gave four fragments. Two of these contained regions essential for replication in *Streptomyces* and one fragment contained *tsr*. The fourth fragment, carrying the *E. coli* replicon and the amino terminus of the *neo* gene from pIJ486, was to be deleted. The *Nco*I-digested plasmid DNA was ligated and then digested with *Sst*I (to eliminate the fourth *Nco*I fragment) before introduction by transformation into "*S. lividans*" protoplasts. Thiostrepton-resistant colonies contained either plasmid DNA of the structure of pIJ688 or plasmids with the *Nco*I fragment containing *tsr* in the opposite orientation. The two plasmid types could easily be distinguished by the sizes of their *Pst*I fragments. *amp*, Ampicillin resistance (from pBR322); P_{lac}, *lacUV5* promoter; *neo'*, *neo* gene truncated at the amino terminus from pKM109-90. See the legend to Fig. 1 for other symbols and a detailed restriction map of pIJ688.

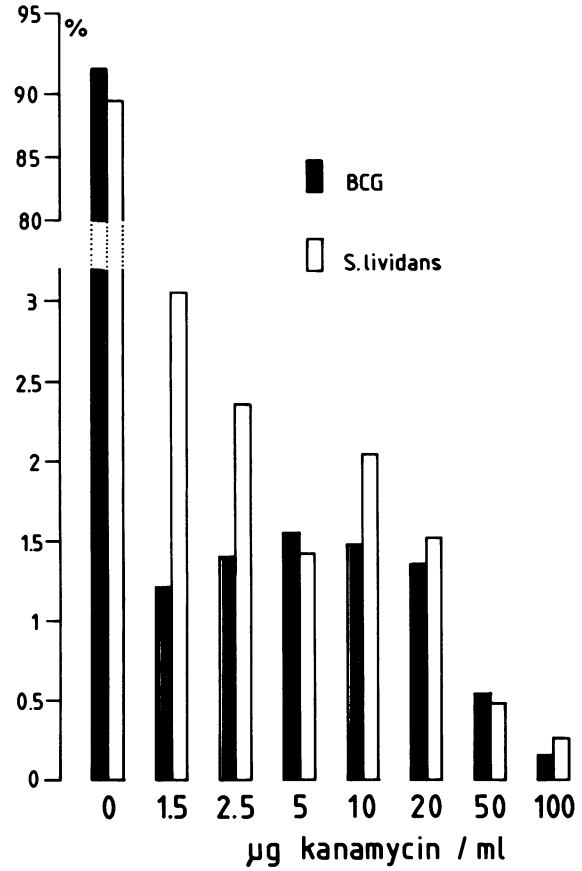


FIG. 5. Kanamycin resistance levels of pIJ688 clones (translational fusion) in "*S. lividans*" TK64 with *Bam*HI fragments of BCG or "*S. lividans*" DNA. Bars indicate the percentages of clones that grew on medium with the indicated concentration of drug but not with the next higher concentration.

observed, showing that the resistance was plasmid-borne. The eight plasmids were also digested with *Bam*HI (to remove the inserts) and religated to regenerate pIJ688. Kanamycin-sensitive "*S. lividans*" transformants were recovered, showing that resistance depended on the cloned BCG DNA.

The maximal expected frequency of active fusions would be one in six (16.6%), assuming that all DNA is coding, that the DNA must be fused in the correct orientation and reading frame, and that every hybrid protein is enzymatically active or degraded to an active form intracellularly. Taking into account the results of the promoter-probing experiment (Fig. 2A), that about 25% of all the clones were kanamycin sensitive and therefore had no detectable promoter activity, one would expect up to 12.5% (75% of 16.6%) of all the clones to show kanamycin resistance. The actual values were close to this figure, suggesting that (nearly) all fusions in the correct reading frame give rise to kanamycin resistance. This interpretation must of course be treated with caution in view of the many assumptions implicit in the calculation. (It should be noted that the placement of the *Bam*HI site with respect to the reading frame in pIJ688 [Fig. 1] favors gene fusion in the correct reading frame, because both "*S. lividans*" and BCG have a high G+C content and thus mostly G or C in the third codon position.)

Having established that kanamycin-resistant colonies carried plasmids with inserts, larger samples of colonies growing on 1.5 µg of kanamycin per ml (316 and 318 clones, respectively, for the experiments with BCG and "*S. lividans*" DNA) were tested on higher levels of kanamycin (Fig. 5). The average resistance level was lower than for the transcriptional fusions in pIJ424. This agrees with the expectation that many of the hybrid proteins would have a lower specific kanamycin phosphotransferase activity than the original protein. There was little difference in the number of resistant clones at different kanamycin concentrations obtained with BCG or "*S. lividans*" inserts except for the two lowest concentrations. This suggests that BCG promoters and translational start signals in their natural relative config-

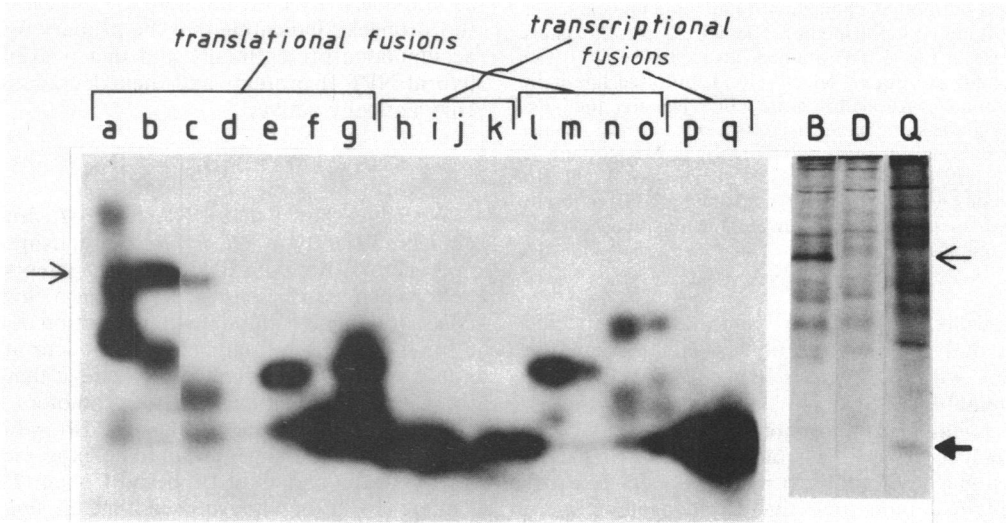


FIG. 6. Detection of kanamycin phosphotransferase activity in lysates of "*S. lividans*" TK64 on a nondenaturing polyacrylamide gel. Lane d, pIJ688 without insert; lanes h through k, p, and q, transcriptional fusions with pIJ424 as vector; lanes a, b, c, e through g, and l through o, translational fusions with pIJ688; lanes B, D, and Q, Coomassie-stained gels with the same extracts as in lanes b, d, and q. The heavy arrow indicates the position of NPT II as produced from the transcriptional fusions. The light arrows indicate the position of the abundant fusion protein in lane B and its activity stain in lane b. All the clones except in lanes d and i contained BCG inserts. Lane i contains an "*S. lividans*" insert.

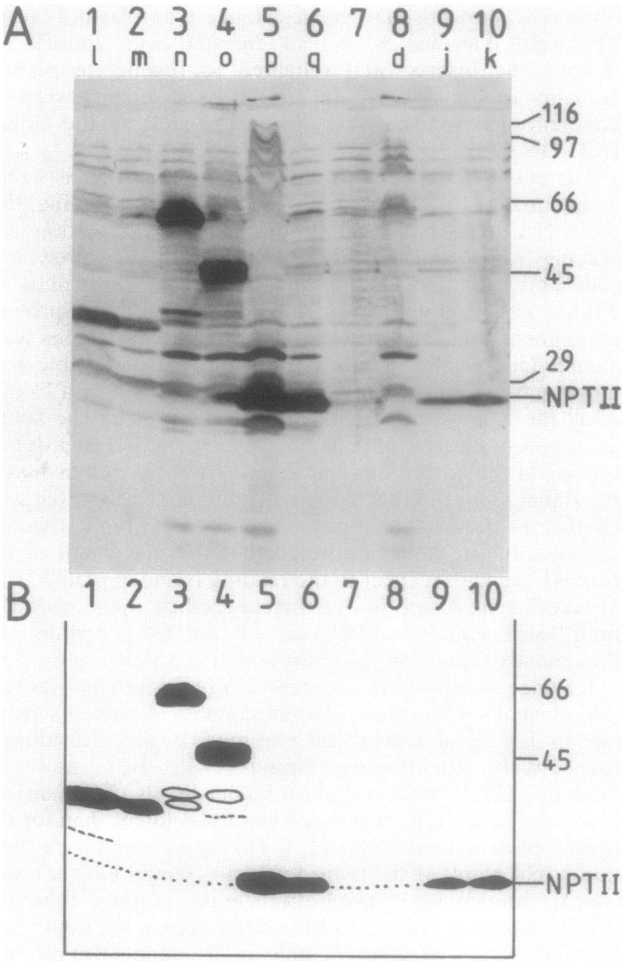


FIG. 7. Detection of kanamycin phosphotransferase in lysates from "*S. lividans*" TK64 with various plasmids, on an SDS-polyacrylamide gel. The gel was blotted to nitrocellulose and the filter was first reacted with rabbit anti-NPT II serum and then with goat anti-rabbit IgG antibodies conjugated to alkaline phosphatase. Staining was with indoxyl phosphate. The letters for the lanes correspond to those in Fig. 6. (A) Stained nitrocellulose filter. (B) NPT II-specific bands are drawn selectively. The dotted line indicates the position of a 27.5-kilodalton protein like the authentic NPT II. There are many nonspecific bands in control lanes 7 and 8. Lanes 1 through 4, Translational fusions of BCG DNA in pIJ688; lane 7, TK64 with pIJ424; lane 8, TK64 with pIJ688; lanes 5, 6, 9, and 10, transcriptional fusions with pIJ424. The bands migrated from top to bottom. The numbers to the right indicate the positions of size markers (in kilodaltons).

uration perform well in "*S. lividans*" and that BCG-specific hybrid mRNAs and proteins are no less stable than the hybrid molecules with "*S. lividans*"-specific sequences at their amino-terminal end.

Detection of kanamycin phosphotransferase activity in nondenaturing polyacrylamide gels (activity blot). Since the translational fusion vector (pIJ688) had not been used before, we needed to demonstrate that fused proteins were indeed generated from the clones in "*S. lividans*." We also wished to confirm that the neomycin phosphotransferase from the transcriptional fusions was identical in size with the authentic enzyme, as predicted from the sequence (Fig. 1) (1), which should preclude the formation of fusion proteins in these constructions. Soluble extracts from highly kanamycin-resistant clones were subjected to electrophoresis on nondenaturing polyacrylamide gels. Phosphotransferase activity was located by the method of Reiss et al. (25). The results (Fig. 6) show, for all transcriptional (pIJ424) fusions, a single band migrating at the same speed as authentic neomycin phosphotransferase (heavy arrow). In contrast, each translational (pIJ688) fusion showed multiple bands, all migrating more slowly than the authentic enzyme. Several of the lysates showed extra bands on the gels stained with Coomassie blue, and these were approximately in the region where the kanamycin phosphotransferase activity was detected. In Fig. 6, particularly strong bands from a transcriptional fusion (heavy arrow, lanes q and Q) and a translation fusion (light arrows, lanes b and B) are shown. Lane D in Fig. 6 shows the banding pattern after Coomassie blue staining obtained with a lysate of "*S. lividans*" containing pIJ688 with no insert. These strong bands indicate that the neomycin phosphotransferase, which originates from the *E. coli* Tn*5*, and at least some of its hybrid derivatives can be highly expressed and stably accumulated in "*S. lividans*."

Detection of kanamycin phosphotransferase in Western blots with specific antibodies. Since the activity blot (Fig. 6) only revealed the positions of enzymatically active proteins, some of the proteins might have been subject to extensive degradation to inactive polypeptides. To clarify this point, extracts of four pIJ424 and four pIJ688 clones containing BCG inserts were subjected to electrophoresis on a denaturing polyacrylamide gel and then blotted to a nitrocellulose filter. The filter was first reacted with rabbit anti-NPT II serum and then with goat anti-rabbit IgG antibodies conjugated to alkaline phosphatase. The developed filter (Fig. 7) showed multiple bands in the control lanes, which may have been due to cross-reacting antibodies in the goat or rabbit serum or to renatured phosphatases from the "*S. lividans*" extracts. The clone-specific bands could, however, easily be identified (and are drawn in Fig. 7B). All the pIJ424 clones gave a single band (the lower part of the gel, which was devoid of specific bands, is not shown in Fig. 7), indicating good stability of this *E. coli* protein in "*S. lividans*." The clones from pIJ688 gave the same number of multiple bands as in the activity blot, but here the largest band was always the strongest, indicating that the primary fusion protein was accumulated preferentially and that possibly all the tested hybrid NPT II proteins and their degradation products are enzymatically active.

DISCUSSION

We have shown that DNA from *M. bovis* BCG can be cloned efficiently in "*S. lividans*" by using multicopy plasmid vectors. Random BCG DNA fragments seem to have a better chance of being transcribed in *S. lividans* than in *E. coli*. Although it is possible that initiation of transcription for a fraction of these fragments might occur at sites which are not promoters in *M. bovis*, we believe that the majority of the active fragments carry true promoters. Our results are consistent with earlier findings (8, 28), which showed that most mycobacterial genes fail to be expressed in *E. coli*, and also with the results of Bibb and Cohen (2) and Jaurin and Cohen (16, 17), who showed that *E. coli* promoters are transcribed when cloned into *Streptomyces* promoter-probe plasmids, even though *Streptomyces* DNA contains few sequences which act as promoters in *E. coli*. One should not ignore the finding that some BCG DNA fragments showed promoter activity in *E. coli*, a result compatible with those already reported (21, 28).

The newly constructed translation fusion vector (pIJ688) was shown to be useful for the generation of enzymatically active (NPT II) hybrid proteins in "*S. lividans*." Analysis of the kanamycin resistance of pIJ688 clones indicated that BCG transcriptional and translational signals compare well with those in the "*S. lividans*" genome. Encouraging for our goal (the cloning and expression of specific *M. leprae* and *M. tuberculosis* genes) is the fact that we could detect clone-specific protein bands in total soluble "*S. lividans*" extracts on Coomassie-stained gels. In some cases these bands could be aligned with kanamycin phosphotransferase-specific bands seen in activity blots or Western blots. This proved that they were proteins expressed from BCG signals rather than chromosomal or plasmid genes which might have been induced indirectly by the presence of the hybrid plasmids, as has been described for *E. coli* by Goff and Goldberg (11), or (unexpectedly) hybrid proteins with a vector-specific amino terminus and a carboxy terminus from the cloned fragment.

The data on gene fusions give only general information about uncharacterized genes and may not hold for each individual gene which might be of (medical) interest. It is possible, however, that *Mycobacterium* genes which are highly expressed in "*S. lividans*" are also highly expressed in their original host and may be good candidates for genes coding for important antigens. It is also possible that pathogenic mycobacteria need to induce specific genes on entering their mammalian host. A likely stimulus is an increase in temperature, as has been shown for parasitic protozoa (30). The heat shock response is a very general phenomenon in pro- and eucaryotes, and temperature induction of mycobacterial genes cloned in "*S. lividans*" might occur.

Other applications, besides the production of antigens, that could profit from a convenient and efficient cloning system in a host capable of expressing mycobacterial genes are the search for exported proteins and for gene clusters which code for biosynthetic pathways. Actinomycetes are known for their ability to produce hundreds of compounds (secondary metabolites), many of which have antibiotic activity, and it has recently been possible to clone in *Streptomyces* species an entire pathway for the biosynthesis of an antibiotic (23). Although no antibiotics have been found in mycobacteria, extracellular glycolipids are common. A further application could be the cloning of resistance determinants from drug-resistant clinical isolates which might be expressed in *Streptomyces* species. The products of these cloned genes could be used to investigate drug resistance mechanisms. This is important because the slow growth of mycobacterial pathogens hinders normal resistance testing.

The *neo* gene from Tn*5* has proved to be a very useful indicator for both transcriptional and translational fusions with high-copy-number *Streptomyces* plasmid vectors. Our results from the quantitative analysis of the frequency and resistance levels obtained with random DNA fragments from total "*S. lividans*" or BCG DNA suggest that even the weakest promoters and most in-frame translational fusions result in a detectable increase in kanamycin resistance. The level of resistance varies over a wide range and, under controlled conditions, can be used as a measure of relative signal strength. These features make pIJ424, pIJ688, and similar gene fusion vectors (31a; Kieser, unpublished) potentially very useful for the study of gene expression in *Streptomyces* spp. The very high frequency (>50%) of random kanamycin-resistant clones also makes it possible to use kanamycin resistance to select plasmids which carry cloned DNA (D. A. Hopwood, M. J. Bibb, K. F. Chater and T. Kieser, Methods Enzymol., in press). With randomly sheared or partially digested target DNA, it might even be possible to achieve nearly complete representation of a whole genome with the benefit of positive selection. This would replace the use of dephosphorylated vectors, which yield fewer recombinant clones.

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LITERATURE CITED

1. **Beck, E., G. Ludwig, E. A. Auerswald, B. Reiss, and H. Schaller.** 1982. Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from transposon Tn*5*. Gene **19**:327–336.
2. **Bibb, M. J., and S. N. Cohen.** 1982. Gene expression in *Streptomyces*: construction and application of promoter-probe plasmid vectors in *Streptomyces lividans*. Mol. Gen. Genet. **187**:265–277.
3. **Bibb, M. J., P. R. Findlay, and M. W. Johnson.** 1984. The relationship between base composition and codon usage in bacterial genes and its use for the simple and reliable identification of protein-coding sequences. Gene **30**:157–166.
4. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
5. **Blake, M. S., K. H. Johnston, G. J. Russell-Jones, and E. C. Gotschlich.** 1984. A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. Anal. Biochem. **136**:175–179.
6. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41**:459–472.
7. **Brosius, J.** 1984. Plasmid vectors for the selection of promoters. Gene **27**:151–160.
8. **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.
9. **Enquist, L. W., and S. G. Bradley.** 1971. Characterization of deoxyribonucleic acid from *Streptomyces venezuelae* spores. Dev. Ind. Microbiol. **12**:225–236.
10. **Gentz, R., A. Langner, A. C. Y. Chang, S. N. Cohen, and H. Bujard.** 1981. Cloning and analysis of strong promoters is made possible by the downstream placement of a RNA termination signal. Proc. Natl. Acad. Sci. USA **78**:4936–4940.
11. **Goff, S. A., and A. L. Goldberg.** 1985. Production of abnormal proteins in *E. coli* stimulates transcription of *lon* and other heat shock genes. Cell **41**:587–595.
12. **Hopwood, D. A., M. J. Bibb, K. F. Chater, G. R. Janssen, F. Malpartida, and C. P. Smith.** 1986. Regulation of gene expression in antibiotic-producing *Streptomyces*, p. 251–276. *In* I. R. Booth and C. F. Higgins (ed.), Regulation of gene expression—25 years on. The Society for General Microbiology, Cambridge University Press, Cambridge.
13. **Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward, and H. Schrempf.** 1985. Genetic manipulation of streptomyces: a laboratory manual. The John Innes Foundation, Norwich, U.K.

14. **Hopwood, D. A., T. Kieser, H. M. Wright, and M. J. Bibb.** 1983. Plasmids, recombination and chromosome mapping in *Streptomyces lividans* 66. J. Gen. Microbiol. **129**:2257–2269.
15. **Hunter, S. W., T. Fujiwara, and P. J. Brennan.** 1982. Structure and antigenicity of the major specific glycolipid antigen of *Mycobacterium leprae*. J. Biochem. **257**:15072–15078.
16. **Jaurin, B., and S. N. Cohen.** 1984. *Streptomyces lividans* RNA polymerase recognizes and uses *Escherichia coli* transcriptional signals. Gene **28**:83–91.
17. **Jaurin, B., and S. N. Cohen.** 1985. Streptomyces contain *Escherichia coli*-type A+T-rich promoters having novel structural features. Gene **39**:191–201.
18. **Kieser, T.** 1984. Factors affecting the isolation of CCC DNA from *Streptomyces lividans* and *Escherichia coli*. Plasmid **12**:19–36.
19. **Kieser, T., D. A. Hopwood, H. M. Wright, and C. J. Thompson.** 1982. pIJ101, a multi-copy broad host-range *Streptomyces* plasmid: functional analysis and development of DNA cloning vectors. Mol. Gen. Genet. **185**:223–238.
20. **Kushner, S. R.** 1978. An improved method for transformation of *Escherichia coli* with ColE1-derived plasmids, p. 17–23. *In* H. B. Boyer and S. Nicosia (ed.), Genetic engineering. Elsevier/North Holland, Amsterdam.
21. **Labidi, A., H. L. David, and D. Roulland-Dussoix.** 1985. Cloning and expression of mycobacterial plasmid DNA in *Escherichia coli*. FEMS Lett. **30**:221–225.
22. **Lennox, E. S.** 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1**:190–206.
23. **Malpartida, F., and D. A. Hopwood.** 1984. Molecular cloning of the whole biosynthetic pathway of a *Streptomyces* antibiotic and its expression in a heterologous host. Nature (London) **309**:462–464.
24. **Reiss, B., R. Sprengel, and H. Schaller.** 1984. Protein fusions with the kanamycin resistance gene from transposon Tn5. EMBO J. **3**:3317–3322.
25. **Reiss, B., R. Sprengel, H. Will, and H. Schaller.** 1984. A new sensitive method for qualitative and quantitative assay of neomycin phosphotransferase in crude cell extracts. Gene **30**:211–218.
26. **Rosenberg, M., and D. Court.** 1979. Regulatory sequences involved in the promotion and termination of RNA transcription. Annu. Rev. Genet. **13**:319–353.
27. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Thole, J. R., H. G. Dauwerse, P. K. Das, D. G. Groothuis, L. M. Schouls, and J. D. A. van Embden.** 1985. Cloning of *Mycobacterium bovis* BCG DNA and expression of antigens in *Escherichia coli*. Infect. Immun. **50**:800–806.
29. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
30. **Van der Ploeg, L. H. T., S. H. Giannini, and C. R. Cantor.** 1985. Heat shock genes: regulatory role for differentiation in parasitic protozoa. Science **228**:1443–1446.
31. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.

31a. **Ward, J. M., G. R. Janssen, T. Kieser, M. J. Bibb, M. J. Buttner, and M. J. Bibb.** 1986. Construction and characterization of a series of multi-copy promoter-probe plasmid vectors for *Streptomyces* using the aminoglycoside phosphotransferase gene from Tn5 as indicator. Mol. Gen. Genet. **203**:468–478.

32. **Westpheling, J., M. Ranes, and R. Losick.** 1985. RNA polymerase heterogeneity in *Streptomyces coelicolor*. Nature (London) **313**:22–27.
33. **Young, D. B., S. R. Khanolkar, L. L. Barg, and T. M. Buchanan.** 1984. Generation and characterization of monoclonal antibodies to the phenolic glycolipid of *Mycobacterium leprae*. Infect. Immun. **43**:183–188.
34. **Young, R. A., B. R. Bloom, C. M. Grosskinsky, J. Ivanyi, D. Thomas, and R. W. Davis.** 1985. Dissection of *Mycobacterium tuberculosis* antigens using recombinant DNA. Proc. Natl. Acad. Sci. USA **82**:2583–2587.
35. **Young, R. A., V. Mehra, D. Sweetser, T. Buchanan, J. Clark-Curtiss, R. W. Davis, and B. R. Bloom.** 1985. Genes for the major protein antigens of the leprosy parasite *Mycobacterium leprae*. Nature (London) **316**:450–452.
36. **Zimmerman, S. B., and B. Harrison.** 1985. Macromolecular crowding accelerates the cohesion of DNA fragments with complementary termini. Nucleic Acids Res. **13**:2241–2249.