Early and Late Responses of TOL Promoters to Pathway Inducers: Identification of Postexponential Promoters in *Pseudomonas putida* with *lacZ-tet* Bicistronic Reporters

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Transcriptional *lacZ* fusions to the *Pu* and *Pm* promoters of the TOL (toluene degradation) plasmid inserted in monocopy in the chromosome of *Pseudomonas putida* showed a very different responsiveness to their respective aromatic effectors regarding growth phase. While a substantial XylS-dependent activation of *Pm-lacZ* was detected nearly instantly after *m*-toluate addition, XylR- and xylene-mediated induction of the σ^{54} promoter *Pu* became significant only after cells slowed down exponential growth and entered stationary phase. When *Pu* and *Pm* were fused to *lacZ-tet* reporters (i.e., promoterless *lacZ* genes coupled to a *tet* gene which confers resistance to tetracycline when cotranscribed with the leading gene) instead of *lacZ* alone, the resulting colonies displayed a distinct phenotype consisting of hyperfluorescence on agar plates after being sprayed with 4-methylumbelliferyl- β -D-galactoside, simultaneously with being either sensitive (*Pu*) or resistant (*Pm*) to tetracycline. To examine whether the same phenotype could be scored in strains carrying transcriptional fusions of the *lacZ-tet* cassettes to other genes or promoters whose expression is silenced during growth and activated in stationary phase, we constructed mini-Tn5 *lacZ-tet* transposons for random genetic probing of promoters preferentially active at advanced stages of growth. Chromosomal insertions of this mobile element were selected by means of the constitutive resistance to kanamycin which is also specified by the transposon. A number of kanamycin-resistant colonies which are hyperfluorescent with 4-methylumbelliferyl- β -D-galactosidase but sensitive to tetracycline and which reached full induction only at postexponential growth stages were obtained.

The TOL plasmid pWW0 of *Pseudomonas putida* encodes two catabolic operons for the complete biodegradation of toluene, *m*-xylene, *p*-xylene (5), the expression of which is subjected to growth phase control superimposed on the primary regulatory circuits mediated by XylR and XylS proteins (7, 8). The σ^{54} -dependent *Pu* promoter activates transcription of the *xylCMABN* upper TOL operon, the products of which transform toluene and xylenes into benzoate and toluates. *Pu* promoter shows a complex regulation (5) which includes growth phase dependency (7). *Pm* promoter drives transcription of the TOL *meta* operon *xylXYZLTEGFJQKIH*, the products of which allow mineralization of benzoate and toluates down to tricarboxylic acid cycle intermediates. Unlike *Pu*, *Pm* becomes activated nearly instantly upon exposure to pathway effectors such as 3-methyl benzoate (*m*-toluate or 3MB) through a mechanism which involves the binding of the XylS activator to cognate *Pm* sequences (9). Transcription from *Pm* is further exacerbated when cells reach stationary phase (7, 8). Using as a model the *Pu* and *Pm* promoters of TOL plasmid and having in mind the early genetic strategies for the identification of sporulation genes in *Bacillus* species (16), we have developed a *lacZ-tet* reporter system for *Pseudomonas* spp. and related bacteria which permits us to distinguish the phenotype endowed by those promoters which are preferentially active at late growth stages from those which are functional along exponential phase. When combined with a mini-Tn5 delivery system, *lacZ-tet* reporters were instrumental as genetic probes for screening promoters which are poorly expressed or silent during the growth phase, reaching maximum activity only after leaving the exponential phase.

MATERIALS AND METHODS

Strains, media, and general methods. *Escherichia coli* CC118 λ pir [Δ (*ara-leu*) *araD* Δ *lacX74* *galE* *galK* *phoA* *thi-1* *rpsE* *rpoB* *argE*(Am) *recA1* λ pir] (6) was used as the host for intermediate plasmid constructions containing an R6K origin of replication. *E. coli* S17-1 λ pir (*recA* *thi* *pro* *hsdR*[−] *M*⁺ RP4::2-Tc::Mu::Km Tn7 Tp^r Sm^r λ pir) is a R6K λ pir phage lysogen of *E. coli* S17-1 (15) and was used in all cases as the delivery strain for mini-Tn5 derivatives. *P. putida* KT2442 is a prototrophic collection strain (6). *P. putida* KT2442 derivatives carrying chromosomal insertions *xylR*⁺/*Pu-lacZ* and *xylS*⁺/*Pu-lacZ* were obtained by mating *E. coli* S17-1 λ pir (pCNB1-*lacZ*) $\times$ *P. putida* KT2442 and *E. coli* S17-1 λ pir (pCNB3-*lacZ*) $\times$ *P. putida* KT2442 as previously described (2). Solid and liquid LB media (13) were supplemented, when required, with 150 μ g of ampicillin (Ap) per ml, 50 to 75 μ g of kanamycin (Km) per ml, 25 μ g of piperacillin (Pip) per ml, or 12 μ g of tetracycline (Tc) per ml. M9 minimal medium (13) was supplemented with 0.2% citrate. Where indicated, selection plates with minimal medium were sprayed with 0.2 ml of a fresh solution of 10 mg of 4-methylumbelliferyl- β -D-galactoside (MUG) per ml in dimethyl sulfoxide. β -Galactosidase (β -Gal) assays were carried out by the method of Miller (13). Insertional mutagenesis of the target strain with mini-Tn5 *lacZ-tet* transposons was by a filter-mating technique (3), with *E. coli* S17-1 λ pir (transformed with pUT derivatives [see Fig. 1]) as the donor strain.

***lacZ-tet* cassettes.** For construction of *lacZ-tet/1*, plasmid pKK175-6 (1) was digested with *Pvu*II and ligated to *Hind*III linkers, after which a derived 1.9-kb *Hind*III fragment containing the *tet* gene of pBR322 with a downstream 5S rRNA terminator was cloned at the *Hind*III sites of pUJ8 (3), in the same orientation as the *trp*'-'*lacZ* gene contained in that

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FIG. 1. Organization of *lacZ-tet* cassettes and related transposons. The elements numbered 1, 2, and 3 correspond to *lacZ-tet/1*, *lacZ-tet/2*, and *lacZ-tet/3*, respectively. Relevant restriction sites and functional elements are indicated in each case: *trp'-lacZ* is a promoterless tryptophan- β -Gal reporter gene fusion from plasmid pUJ8 (3), whereas *lacZ* is a wild-type-size β -Gal gene, and *'lacZ* is a truncated derivative devoid of the first eight codons of its structural sequence. Transcription of the *lacZ-tet* units occurs right to left. In each element, a strong 5S terminator is located downstream of the *tet* (*Tc'*) gene. Insertion of the *lacZ-tet* cassettes at compatible *NotI* (elements 1 and 2) or *SfiI* (element 3) sites of mini-Tn5 Km transposons (as shown on top and bottom of the figure) results in three new hybrid mobile elements instrumental in the identification of growth-phase-dependent promoters in *P. putida* and related bacteria (see text). Gene fusions between a target gene and the *lacZ-tet* units are generated by transposition mediated by the 19-bp O and I ends of Tn5 (6). For its delivery into a target strain, the mini-Tn5 *lac tet* elements are present on donor plasmid pUT (6). Elements 1 and 2 create type I fusions (transcriptional), whereas element 3 is used to construct type II fusions (translational).

plasmid. This step generated a *NotI* fragment which contained a promoterless *trp'-lacZ-tet* tandem. The *lacZ-tet/2* cassette is identical to *lacZ-tet/1*, except that the *BamHI*-*ClaI* fragment of the *trp'-lacZ* gene has been substituted by an equivalent portion of pKOK6 plasmid (10), which reconstructs the 5' sequence of the *lacZ* gene along with its ribosomal binding site. *lacZ-tet/3* was constructed as an *SfiI* restriction fragment. For this, the *EcoRI*-*HindIII* portion of pUJ9 (3) containing a truncated *'lacZ* sequence was cloned at the corresponding sites of p18Sfi (6) and added downstream with the *tet* gene mentioned above, at its *HindIII* site.

Construction of chromosomal *Pm-lacZ-tet* and *Pu-lacZ-tet* fusions. To generate *P. putida* KT2442 derivatives carrying transcriptional fusions of a *lacZ-tet* cassette to TOL promoters, we started by cloning the *lacZ-tet/2* cassette (Fig. 1) as a *NotI* restriction fragment at the unique *NotI* site of plasmids pCNB1 (for *Pm-lacZ-tet*) and pCNB3 (for *Pu-lacZ-tet*). These two plasmids (2) are pUT derivatives (6) in which expression cassettes containing either the *xylS/Pm* or *xylR/Pu* elements of the TOL plasmid (5) have been engineered within the boundaries of a mini-Tn5 transposon containing either *Km*^r (pCNB1)

or *Sm*^r (pCNB3) selection determinants. Since such minitransposons contain the *Pm* and *Pu* catabolic TOL promoters pointing towards a single *NotI* site, *lacZ-tet/2* inserts in each of the plasmids created two hybrid minitransposons: mini-Tn5 *xylS/Pm-lacZ-tet* and mini-Tn5 *xylR/Pu-lacZ-tet*. The corresponding suicide delivery plasmids (6) were named pVDL20 (or pCNB1-*lacZ-tet/2*) and pVDL22 (or pCNB3-*lacZ-tet/2*). These minitransposons were transferred to the chromosome of *P. putida* KT2442 as previously described (3), resulting in the insertions represented in Fig. 3.

Mini-Tn5 *lacZ-tet* transposons. Mini-Tn5 *lacZ-tet/1* and mini-Tn5 *lacZ-tet/2* were constructed by cloning the *lacZ-tet/1* and *lacZ-tet/2* cassettes (Fig. 1) as *NotI* restriction fragments at the unique *NotI* site of the mini-Tn5 Km2 transposon (3). The delivery plasmids for these mobile elements are named pVDL24 (or pUT/mini-Tn5 *lacZ-tet/1*) and pVDL21 (or pUT/mini-Tn5 *lacZ-tet/2*). Mini-Tn5 *lacZ-tet/3* was made by cloning the *lacZ-tet/3* cassette (Fig. 1) as an *SfiI* restriction fragment at the unique *SfiI* site of pUT/Km (6). The resulting delivery plasmid is called pUJ29 (or pUT/mini-Tn5 *lacZ-tet/3*).

RESULTS AND DISCUSSION

Early and late responses of TOL promoters to pathway inducers. In order to devise a general system to identify promoters of *P. putida* which are preferentially expressed at late growth stages, we first examined whether the well-characterized *Pu* and *Pm* promoters of the TOL plasmid could be used as prototypes of growth-phase-dependent and growth-phase-independent systems, respectively. Previous results (7) had indicated that expression of *Pu* is subjected to some type of growth phase and/or catabolite control, since induction of upper-TOL pathway enzymes in the presence of effectors is virtually negligible when cells grow exponentially in rich medium. On the contrary, the *Pm* promoter seems to respond very quickly to meta pathway inducers (8). To assess whether *Pu* and *Pm* provide a reference for the two types of performance in our assay conditions, we analyzed in detail the induction pattern of *P. putida* strains carrying chromosomal insertions of hybrid mini-Tn5 transposons in which *xylR*⁺/*Pu-lacZ* or *xylS*⁺/*Pm-lacZ* cassettes had been engineered (2). As shown in Fig. 2, the behavior of each promoter is clearly different after cultures were exposed to their respective inducers. Cells with the *Pu-lacZ* fusions accumulated β -Gal only after the culture had reached an optical density at 600 nm of 1.0 to 1.2, beyond which the levels were quickly raised >100-fold. On the contrary, a significant induction of *Pm* was detected very shortly after addition of 3MB. These results provided the reference sought to devise a distinct, scorable phenotype endowed by a reporter system to the two types of promoters.

Phenotypes associated with *Pu* and *Pm* promoters fused to *lacZ-tet* cassettes. Our approach to scoring promoters which are preferentially active at advanced growth stages in *P. putida* was similar to that developed in Losick's laboratory (16) to detect sporulation genes in *Bacillus* species. Each of the three *lacZ-tet* cassettes shown in Fig. 1 consists of a promoterless bicistronic operon composed of a *lacZ* gene with different 5' ends fused transcriptionally to a *Tc*^r gene (*tet* [1]). The criterium to identify promoters active at low growth rates assumes that in a fully grown colony, much of the cell population is in the stationary phase. Colonies actively expressing β -Gal as the result of the gene fusion to such promoters are predicted to give strong fluorescence with UV light upon spraying the plate with MUG, a fluorescent substrate of β -Gal (see Fig. 4). However, postexponential promoters should prevent formation of colonies in a medium with tetracycline, because early growth silences the expression of both *lacZ* and *tet* genes.

To validate the predictions of phenotypes associated with *lacZ-tet* reporters, we placed one of the bicistronic cassettes (*lacZ-tet/2*) within a mini-Tn5 transposon downstream of either *Pu* or *Pm* sequences along with their cognate regulatory genes, *xylR* and *xylS*, respectively, and then we inserted the whole unit into the chromosome of *P. putida* KT2442 (Fig. 3). As in the case of the *tet*-less counterparts of Fig. 2, this reproduced faithfully in monocopy gene dosage all the regulatory elements which control *Pm* and *Pu* promoter activities. Strains carrying each of the hybrid transposons with the *lacZ-tet* fusions were plated on media carrying their respective inducers, and they were examined for (i) fluorescence of well-grown colonies upon spraying with MUG and (ii) growth on medium with tetracycline. As summarized in Fig. 3, in induced conditions both types of insertions gave rise to colonies which were hyperfluorescent with MUG when exposed to their respective effectors. However, while the strain carrying a *lacZ-tet* fusion to *Pm* could form colonies when streaked out on tetracycline plates, the counterpart with *Pu* could not. There-

FIG. 2. Induction profiles of *Pu* and *Pm* promoters upon exposure to TOL pathway effectors. The drawings on top of the graphs (not to scale) represent the structures of the hybrid mini-Tn5 elements containing transcriptional fusions *Pu-lacZ* and *Pm-lacZ* inserted into the chromosome (zigzag lines) of the *P. putida* KT2442 derivatives under study. *Sm*^r and *Km*^r selection markers present in each of the transposons, as well as the position of the *I* and *O* ends of the transposons, and the direction of transcription of the different elements, are indicated. For induction experiments, *P. putida* KT2442::mini-Tn5 *xylR*/*Pu-lacZ* (top) was grown at 30°C in Luria-Bertani medium up to an optical density at 600 nm of 0.1 and exposed to saturating vapors of upper-TOL pathway inducer *m*-xylene. After that moment, samples were taken every half hour for the first 5 h, and then every hour up to 10 h, and finally overnight. *P. putida* KT2442::mini-Tn5 *xylS*/*Pm-lacZ* strain (bottom) was induced in the same way, except that cultures were added with 3 mM meta-TOL pathway inducer 3MB. In both cases, the values of β -Gal were obtained as double points in experiments carried out during 3 different days. The activity of *Pu* and *Pm* is negligible in the absence of inducers (not shown).

fore, sensitivity to tetracycline permitted differentiation of promoters whose activity is triggered immediately after inducer addition (early response, *Pm*) from promoters being active only after some time of exposure to its effector (late response, *Pu*). If the lag for the onset of *lacZ-tet* expression is long enough (as is the case for growth-phase-dependent promoters), it is predicted that the tetracycline present in the medium will inhibit growth regardless of the final levels of β -Gal reached after full induction. Therefore, hyperfluorescence and sensitivity to tetracycline of the strain carrying the *Pu-lacZ-tet* insert (Fig. 3) reflect faithfully the delay in the expression of the genes of the TOL upper pathway which

FIG. 3. Phenotypes resulting from *lacZ-tet* fusions to *Pm* and *Pu* promoters of TOL plasmid. The drawings on top of the boxes represent the mini-Tn5 elements containing transcriptional fusions *Pm-lacZ-tet/2* and *Pu-lacZ-tet/2* in the chromosome of the corresponding *P. putida* KT2442 derivatives. For qualitative detection of fluorescence, the strains were streaked out on minimal-citrate plates with either 0.5 mM 3MB for *Pm* induction or 2 mM soluble *Pu* inducer *p*-chloro benzyl alcohol and sprayed with MUG as explained in Materials and Methods (for a visual estimate of different degrees of fluorescence, see Fig. 4). Resistance to tetracycline was determined by patching hyperfluorescent colonies on minimal-citrate plates with 2 mM *p*-chloro benzyl alcohol and 12 μ g of the antibiotic added per ml.

occurs when cells growing exponentially on other carbon sources are exposed to TOL inducers (see above) (7).

Probing exponential and postexponential promoters with mini-Tn5 *lacZ-tet* transposons. Mini-Tn5 *lacZ-tet* transposons are mobile elements which contain a constitutively expressed *Km^r* gene from Tn903 and one each of the *lacZ-tet* cassettes shown in Fig. 1. This group of determinants is flanked by the two 19-bp O and I termini of Tn5, and their mobility is based on the presence in *cis* of the IS50R *tnp* gene in the pUT-type suicide delivery plasmid (6), in which the different elements have been engineered. Since a *bla* gene conferring resistance to β -lactams is present in pUT (6), *Km^r* *Pip^s* exconjugants of *P. putida* (and potentially other gram-negative bacteria as well) thus represent recipients carrying the mini-Tn5 *lacZ-tet* element as random stable inserts lacking the transposase gene (*tnp*) and the rest of the pUT plasmid. When inserted in the appropriate orientation, mini-Tn5 *lacZ-tet* transposons yield transcriptional (mini-Tn5 *lacZ-tet/1* and mini-Tn5 *lacZ-tet/2*) or translational (mini-Tn5 *lacZ-tet/3*) fusions of *lacZ-tet* with target genes. The choice between mini-Tn5 *lacZ-tet/1* and mini-Tn5 *lacZ-tet/2* to generate transcriptional fusions depends on the specific microorganism under study, since the basal level of β -Gal produced by the leading promoterless *lacZ* genes seems to vary among strains and genera (11).

To detect transposon insertions downstream of promoters preferentially induced at late growth phase, well-grown exconjugant colonies of *P. putida* KT2442 were sprayed with MUG as described in Materials and Methods (Fig. 4), and those giving a strong fluorescence under UV light were scored for resistance to tetracycline. Since colony formation means active growth in the presence of the antibiotic, *Tc^r* exconjugants presumably have insertions downstream of promoters functioning in logarithmic as well as stationary phases, whereas *Tc^s* colonies have, in principle, the *lacZ-tet* reporter downstream of promoters silenced during early growth stages. In our hands, after mutagenesis with the mini-Tn5 *lacZ-tet/1* element, *Km^r*

FIG. 4. Probing growth-phase-dependent promoters of *P. putida* KT2442 with mini-Tn5 *lacZ-tet* transposons. Donor strain *E. coli* S17-1 λ pir (pUT/mini-Tn5 *lacZ-tet/1*) was mated with recipient strain *P. putida* KT2442 as previously described (3), and after overnight incubation, the conjugation mixture was plated on M9-citrate medium with 70 μ g of kanamycin per ml (nutritional selection in minimal medium is preferred over counterselection of the donor strain in rich medium, since it minimizes background growth and MUG fluorescence). The structure of the mini-Tn5 *lacZ-tet* element is schematized at the top. The plates were grown at 30°C for 2 days to let *P. putida* colonies reach their full size, approximately 2 mm in diameter. The colonies were then sprayed with a solution of MUG. After 2 to 5 min, the colonies were placed on top of a 366-nm UV transilluminator and photographed with Polaroid type 667 or similar film under the same standard conditions used to obtain ethidium bromide-stained DNA gels. For a colored record, plates can be illuminated from above with 366-nm UV light, and Polaroid pictures (Polaroid film type 669) are then taken with a Kodak 2E filter. Hyperfluorescent colonies may arise from expression of *lacZ* at late stages of growth or from accumulation of β -Gal during growth. To distinguish between the two possibilities, colonies were then scored for tetracycline resistance (see text).

Pip^s *P. putida* exconjugants were obtained with a frequency of 10^{-6} to 10^{-7} . A total of 100 colonies arising from different matings and showing strong fluorescence with MUG were then scored for resistance to tetracycline by being patched on tetracycline plates. Ten of these were *Tc^s* and were subjected to a liquid culture assay of β -Gal levels versus cell growth. Figure 5 shows several of the resulting β -Gal courses. As expected, most of those inserts appear to have happened in genes which are preferentially expressed when cells have left exponential growth (i.e., after optical density at 600 nm is >1.0).

Although we have chosen resistance to tetracycline as the second selection determinant because it is a very useful marker in *P. putida* and related bacteria, the modular nature of the transposons (Fig. 1) does permit exchange of the *tet* gene by any other marker which may be more useful in other bacterial species or which may actually be counterselected. It should be noted, however, that some insertion mutants might not be viable in haploid state, and severe carbon starvation may eventually prevent biosynthesis of the reporter enzyme. Although this random procedure does not discriminate between different mechanisms accounting for the onset of β -Gal activity at advanced stages of growth (12, 14), stationary-phase pro-

FIG. 5. Late-growth-phase induction of *P. putida* genes revealed by the mini-Tn5 *lacZ-tet/l* transposon. *P. putida* exconjugants with a phenotype indicative of mini-Tn5 *lac tet* insertion in growth-phase-regulated genes (see text) were cultured at 30°C in LB medium in batch culture and analyzed for β -Gal activity (13). Note maximal levels of β -Gal at postexponential growth stages of exconjugants designated PP16, PP5, PP9, PP11, and PP6. PP67 is a control exconjugant with an insertion in a low-constitutive-expression gene.

motors may have interesting applications for heterologous expression purposes in genetically engineered microorganisms destined for environmental release which may need to function under nutrient starvation and/or very low growth rates.

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REFERENCES

1. Brosius, J., and J. R. Lupski. 1987. Plasmids for the selection and analysis of prokaryotic promoters. *Methods Enzymol.* **153**:54–68.
2. de Lorenzo, V., S. Fernández, M. Herrero, U. Jakubzik, and K. N. Timmis. Engineering of alkyl- and halo-aromatic-responsive gene expression with mini-transposons containing regulated promoters of biodegradative pathways of *Pseudomonas*. *Gene* **130**:41–46.
3. de Lorenzo, V., M. Herrero, U. Jakubzik, and K. N. Timmis. 1990. Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. *J. Bacteriol.* **172**:6568–6572.
4. de Lorenzo, V., M. Metzke, M. Herrero, and K. N. Timmis. 1991. An upstream XylR and IHF-induced nucleoprotein complex regulates the σ^{54} -dependent *Pu* promoter of TOL plasmid. *EMBO J.* **10**:1159–1167.
5. Harayama, S., and K. N. Timmis. 1989. Catabolism of aromatic hydrocarbons in *Pseudomonas*, p. 151–174. In D. Hoopwood and K. Chater (ed.), *Genetics of bacterial diversity*. Academic Press, London.
6. Herrero, M., V. de Lorenzo, and K. N. Timmis. 1990. Transposon vectors containing non-antibiotic selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J. Bacteriol.* **172**:6557–6567.
7. Hugouvieux, N., T. Köhler, M. Rekik, and S. Harayama. 1990. Growth-phase-dependent expression of the *Pseudomonas putida* TOL plasmid pWW0 catabolic genes. *J. Bacteriol.* **172**:6651–6660.
8. Kessler, B., V. de Lorenzo, and K. N. Timmis. 1992. A general system to integrate *lacZ* fusions into the chromosomes of Gram-negative eubacteria: regulation of the *Pm* promoter of the TOL plasmid studied with all controlling elements in monocopy. *Mol. Gen. Genet.* **233**:293–301.
9. Kessler, B., V. de Lorenzo, and K. N. Timmis. Identification of a *cis*-acting sequence within the *Pm* promoter of the TOL plasmid which confers XylS-mediated responsiveness to substituted benzoates. *J. Mol. Biol.* **230**:699–703.
10. Kokotek, W., and W. Lotz. 1989. Construction of a *lacZ*-kanamycin resistance cassette useful for site-directed mutagenesis and as a promoter probe. *Gene* **84**:467–471.

11. **Labes, M., A. Pühler, and R. Simon.** 1990. A new family of RSF1010-derived expression and *lac*-fusion broad host range vectors for Gram-negative bacteria. *Gene* **89**:37–46.
12. **Matin, A., E. Auger, P. H. Blum, and J. E. Schulz.** 1989. Genetic basis of starvation survival in nondifferentiating bacteria. *Annu. Rev. Microbiol.* **43**:293–316.
13. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Mulvey, M. R., and P. C. Loewen.** 1989. Nucleotide sequence of *katF* of *Escherichia coli* suggests KatF protein is a novel σ transcription factor. *Nucleic Acids Res.* **17**:9979–9991.
15. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. *Bio/Technology* **1**:784–791.
16. **Youngman, P., P. Zuber, J. B. Perkins, K. Sandman, M. Igo, and R. Losick.** 1985. New ways to study developmental genes in spore-forming bacteria. *Science* **228**:285–291.