Reversion of an *E. coli* strain carrying an IS*1*-activated *bgl* operon under nonselective conditions is predominantly due to deletions within the structural genes

ANITA YAKKUNDI*, SUDHA MOORTHY* and S. MAHADEVAN†

Department of Molecular Reproduction, Development and Genetics, Indian Institute of Science, Bangalore 560 012, India

Abstract

The *bgl* operon of *Escherichia coli*, which encodes the genes necessary for transport and catabolism of β-glucosides, is silent in wild-type cells and is activated by the transposition of IS elements. The silent form of the operon appears to be the stable state. We isolated Bgl⁻ revertants of an activated strain after growth under nonselective conditions to understand whether activation of the cryptic operon by IS elements is reversible. Genetic and molecular analyses revealed that a majority of revertants carry deletions of the *bgl* structural genes, indicating that an irreversible alteration has occurred in the operon. Implications of these results for the evolution and maintenance of cryptic genes are discussed.

[Yakkundi A., Moorthy S. and Mahadevan S. 1998 Reversion of an *E. coli* strain carrying an IS*1*-activated *bgl* operon under nonselective conditions is predominantly due to deletions within the structural genes. *J. Genet.* **77**, 21–26]

Introduction

Cryptic genes have been defined as phenotypically silent genes that are not expressed during the normal life cycle of an organism (Hall *et al.* 1983). Unlike pseudogenes that carry a large number of inactivating mutations, cryptic genes can be activated by a single genetic event such as mutation, recombination or transposition. Their presence is evident only upon mutation, which results in a detectable phenotype. They are nevertheless maintained over generations and therefore their existence is enigmatic in terms of function and evolution (for a review see Mukerji and Mahadevan 1997a).

One of the models for the retention of cryptic genes by microorganisms suggests that they are maintained through repeated cycles of activation and cryptification depending on environmental conditions (Hall *et al.* 1983). The presence of an active allele may be disadvantageous in one environment, and the cryptic state is therefore selected for. However, in another environment (such as presence of a particular substrate), the active allele may be advantageous for growth of the organism. Then the cryptic state would be a transient state and oscillations between cryptic and active states could provide a mechanism to meet changes in the environment, which impose different fitness values on the two states.

The *bgl* operon of *Escherichia coli* is one of the well-studied cryptic genetic systems. It specifies the genes required for utilization of aromatic β-glucosides such as salicin and arbutin (Prasad and Schaefler 1974). Wild-type cells, however, remain Bgl⁻ because of a low level of expression of the *bgl* genes. The operon is activated by a variety of mutations such as insertions of IS*1* or IS*5*, point mutations in the CAP–cAMP binding region upstream of the promoter, and unlinked mutations in the *gyrA, gyrB, hns* and *bglJ* genes (Reynolds *et al.* 1981, 1986; DiNardo *et al.* 1982; Higgins *et al.* 1988; Schnetz and Rak 1992; Giel *et al.* 1996). Transcriptional activation by the different classes of mutations is brought about by disruption of negative elements near the promoter (Lopilato and Wright 1990; Schnetz 1995; Singh *et al.* 1995; Mukerji and Mahadevan 1997b). Upon activation, the *bgl* genes are also

*These authors made an equal contribution to the work.
†For correspondence. E-mail: mahi@serc.iisc.ernet.in.

Keywords. cryptic genes; β-glucoside utilization; microbial evolution.

subject to regulation involving antitermination of transcription (Mahadevan and Wright 1987; Schnetz and Rak 1988; Houman *et al.* 1990; Amster-Choder and Wright 1992). The presence of a silent promoter, despite a sophisticated mechanism for gene regulation, is an evolutionary puzzle.

The present study was undertaken to understand why the *bgl* genes are maintained in a cryptic state and whether cycling might occur between the cryptic and active states when cells are subjected to different growth conditions. If the cryptic allele is to be stably maintained in a population, the activating mutation must be reversible under nonselective conditions. To test this possibility, Bgl^- revertants of an insertionally activated Bgl^+ strain, isolated under conditions nonselective for *bgl* expression, were subjected to genetic and molecular analysis to determine the nature of the reversion. These analyses show that the revertants carry large deletions of the structural genes, which suggests that cycling between cryptic and active states of the *bgl* operon is not likely to occur under laboratory conditions.

Materials and methods

Strains and plasmids: The strains and plasmids used in this study are listed in table 1. Isolation of the revertants and construction of the plasmid pMN25-R3 are described below.

Isolation of Bgl⁻ revertants: For isolation of Bgl^- revertants, cultures of AE328 (*bglR*::IS*1*, Bgl^+) were grown in LB medium for 14–16 hours with aeration. The cultures were suitably diluted and plated on MacConkey agar indicator medium supplemented with 1% salicin. White colonies were streaked on a fresh indicator plate to confirm the phenotype. Isolated single colonies were used to seed cultures for detailed characterization of the revertants.

Complementation analysis: Bgl^- revertants were transformed with the ampicillin-resistant plasmids p1H (*bglF*$^+$ *bglB*$^+$) and pMN25 (*bglR25 bglG*$^+$). The phenotype of ampicillin-resistant transformants was checked on MacConkey–salicin indicator plates. Strains showing complementation were identified as having structural gene mutations.

Southern hybridization: All DNA manipulations and Southern hybridizations were performed as described previously (Sambrook *et al.* 1989). A 1.2-kb *Ssp*I fragment from the plasmid pMN300 was used as probe.

PCR analysis: Oligonucleotide primers used to amplify genomic DNA from revertants and map the end points of deletions in the revertants are listed below:

SM1 (forward, −250):
5′GTGGATCCATCTTCTACTACGTGAAG3′
SM2 (reverse, +340):
5′AGGAATTCGACTTAAGAGTTCGCTTA3′
SM26 (reverse, +1092):
5′CCCTCTACCGCTTTGCGG3′
SM29 (forward, +97of IS*1*):
5′AGTAGCTGAACAGGAGGG3′

The conditions for PCR amplification were: initial denaturation at 95°C for 10 minutes; 30 cycles of denaturation at 95°C for 1 minute, annealing at 50°C for 1 minute and extension at 72°C for 2 minutes; and a final extension at 72°C for 5 minutes.

Conditions for PCR sequencing of the revertants to determine deletion end points were: initial denaturation at 95°C for 5 minutes; and 30 cycles of denaturation at 95°C for 30 seconds, annealing at 54°C for 30 seconds and extension at 70°C for 60 seconds. The products were separated on a 6% acrylamide–urea gel and subjected to autoradiography.

Results

Isolation and genetic characterization of Bgl⁻ revertants

As the *bgl* operon (figure 1) is silent in wild-type strains, this may be the stable state of the operon under nonselective conditions. If cycling between active and silent states is

Table 1. Genotypes of strains and plasmids used in this study.

Strain or plasmid	Description	Source or reference
Bacterial strains		
AE328	Δ*lacX74 thi bglR11* (*bglR*::IS*1*), *tna*::Tn*10* (Bgl^+)	A. Wright
MM1	F^- Δ*lacX74 thi bglR*^{0} *tna*::Tn*10* (Bgl^-)	M. Mukerji
RV^+	F^- Δ*lacX74 thi bglR11*(*bglR*::IS*1*) (Bgl^+)	Mahadevan *et al.* 1987
SM1	RV^+ *rpoS*::Tn*10*	This work
328R1-328R502	Spontaneous revertants of AE328 (Bgl^-)	This work
Plasmids		
p1H	*bglG*′ *bglF*$^+$ *bglB*$^+$ Ap^r	Mahadevan *et al.* 1987
pMN25	*bglR25 bglG*$^+$ *bglF*′ Ap^r	Mahadevan *et al.* 1987
pMN300	*bglR*^{0} *bglG*$^+$ *bglF*$^+$ *bglB*$^+$ Ap^r	Singh *et al.* 1995
pMN22AE	*bglR*::IS*1 bglG*$^+$ *bglF*$^+$ *bglB*$^+$ Ap^r	Singh *et al.* 1995
pMN25-R3	*bglR3 bglG*$^+$ *bglF*′ Ap^r	This work

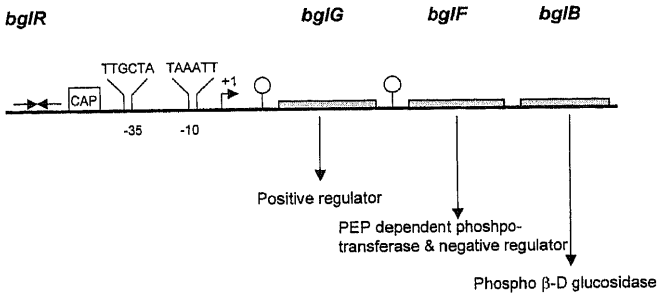

Figure 1. Organization of the *bgl* operon in *E. coli.* The structural genes are shown as shaded boxes. Features of the regulatory region such as the promoter and CAP site are indicated within the region marked *bglR*. One of the negative elements is shown as an inverted repeat. The transcription start is indicated by the right-angle arrow. The stuctures flanking *bglG* are rho-independent terminators involved in regulation mediated by the *bglG* and *bglF* gene products.

indeed possible, most of the revertants obtained after prolonged growth under nonselective condition are expected to be those in which the original activating mutation has undergone a true reversion. To test this possibility, we grew the Bgl^+ strain AE328, which carries an IS*1* insertion in *bglR* (*bglR*::IS*1*), under nonselective conditions for several generations and isolated Bgl^- revertants as white colonies on indicator plates containing salicin. Bgl^- revertants were detected at a frequency of about 10^{-5}.

To test whether any of the revertants carry structural-gene mutations, we subjected them to complementation analysis using plasmids carrying the *bgl* structural genes. Of the 18 revertants studied, only three carried mutations in structural genes as seen by complementability by plasmids carrying the structural genes (table 2). The remaining 15 revertants also seemed to differ from the wild-type Bgl^- *E. coli* strains. They were unable to papillate on MacConkey–salicin plates, suggesting that they are also not true revertants.

The revertants carry large deletions within the operon

The observation that the Bgl^- revertants cannot be activated again to give a Bgl^+ phenotype suggested that the revertants carry a permanent rearrangement of the *bgl* genes. This was confirmed by our inability to amplify the *bgl* regulatory region by PCR using primers flanking the *bglR* region. To determine the status of the *bgl* structural genes in the revertants, genomic DNA from the revertants was subjected to Southern analysis using a probe spanning the *bglR–bglG* region. The results, presented in figure 2, indicate that the revertants carry large deletions within the structural genes.

Digestion of genomic DNA with *Eco*RV results in two bands corresponding to the *bgl* operon (figure 2A). The *bglR–bglG'* region gives rise to a 2.6-kb band in IS*1*-activated strains such as AE328 and a 1.9-kb band in wild-type strains such as MM1. Both strains also give rise to a 2.3-kb band corresponding to the downstream sequences.

Table 2. Complementation analysis of Bgl^- revertants of AE328.

Strain	Papillation*	Complementation*	
		p1H (*bglF*$^+$*bglB*$^+$)	pMN25(*bglR25 bglG*$^+$)
328-R1	–	–	–
328-R2	–	–	–
328-R3	–	–	–
328-R4	+	+	–
328-R101	NA	–	+
328-R103	–	–	–
328-R104	–	–	–
328-R105	–	–	–
328-R106	–	–	–
328-R107	–	–	–
328-R108	–	–	–
328-R204	–	–	–
328-R205	–	–	–
328-R301	–	–	–
328-R302	–	+	–
328-R303	–	–	–
328-R501	–	–	–
328-R502	–	–	–

*Complementation and papillation were checked on MacConkey agar plates supplemented with 1% salicin.
NA, Not applicable as the revertant had a leaky phenotype and the colonies turned pink after 12 hours of incubation.

The signals obtained for DNA from the revertants did not correspond to those for either the wild-type or the IS-activated allele. In most of the revertants (R3, R103–R107, R204, R205 and R301) a single band of ~4.0 kb was observed (figure 2B). Since the expected fragment size due to a mutation resulting in the loss of the *Eco*RV site within *bglG* would be ~4.9 kb for the activated strain, this indicated the presence of a 1.0-kb deletion spanning the *Eco*RV site at +492. Revertant R1 gave a single band of ~3.8 to 3.6 kb indicating a larger deletion, and R2 seemed to have still larger deletion of about 2.5 kb as indicated by the band size of 2.4 kb.

The extent of the deletions in R3, R103–R107 and R301 was confirmed by PCR amplification using the forward primer SM1 and a reverse primer SM26 corresponding to a location further downstream to the *Eco*RV site at +492 (figure 2A). It was apparent that the products obtained in the case of the revertants were smaller in size (1 kb) compared to the PCR product of 2.0 kb seen in the case of the activated strain (data not shown). These observations indicated a 0.9 to 1.0 kb deletion in the revertants. These results are consistent with the results of the Southern analysis.

Molecular analysis of the revertants

To determine the extent of the deletions in the revertants, we employed the following strategy. Initially the revertant AE328-R3 was transformed with the plasmid pMN25 which carries *bglR25* and *bglG*$^+$. Transformants were allowed to papillate on salicin indicator medium to select for recombination between the chromosomal and plasmid alleles (figure 3). Plasmid DNA isolated from papillae was used to

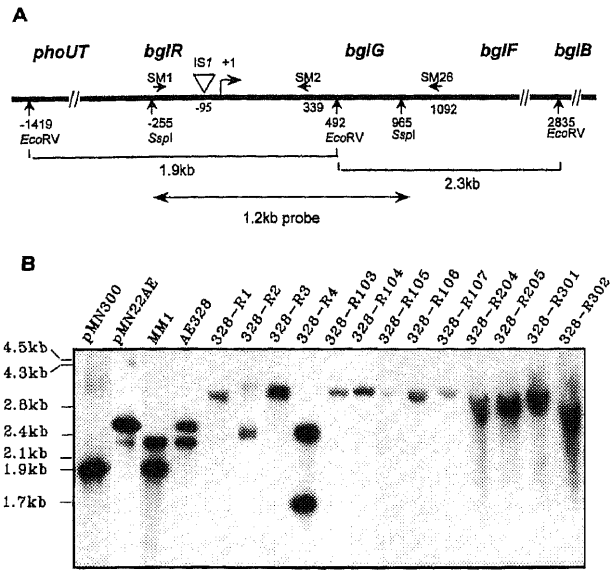

Figure 2. (A) Restriction map of the *bgl* genes. The positions of the IS*1* insertion and PCR primer binding regions used in this study are indicated. (B) Southern hybridization analysis of *bglR* and *bglG* regions of Bgl$^-$ revertants. Genomic DNA from AE328 (*bglR*::IS*1*) and MM1 (*bglR*^{0}) were used as controls. DNA was digested with *Eco*RV and was probed with a 1.2-kb *Ssp*I fragment carrying the *bglR–bglG* region of the operon.

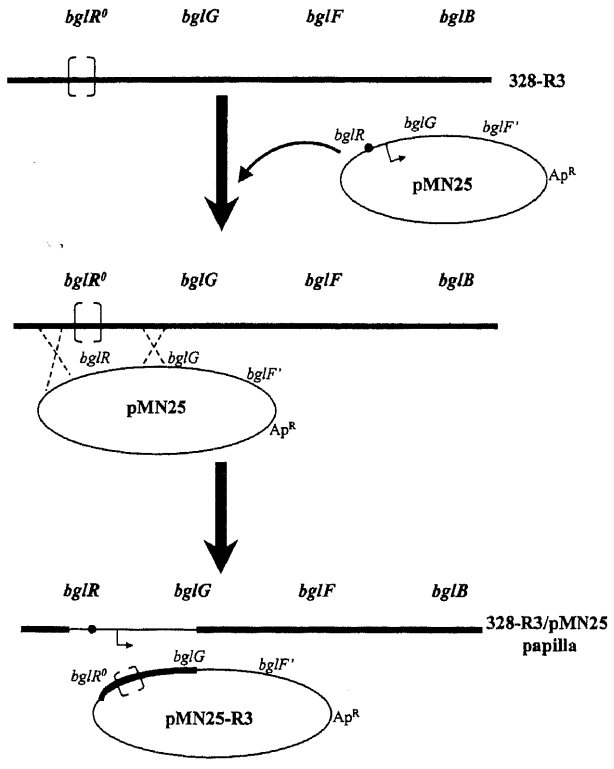

Figure 3. Schematic representation of isolation of pMN25-R3 from 328-R3/pMN25 papilla.

tranform a *bglG* mutant. DNA was isolated from transformants that did not show complementation and therefore presumably carried the *bglR* allele originally from AE328-R3

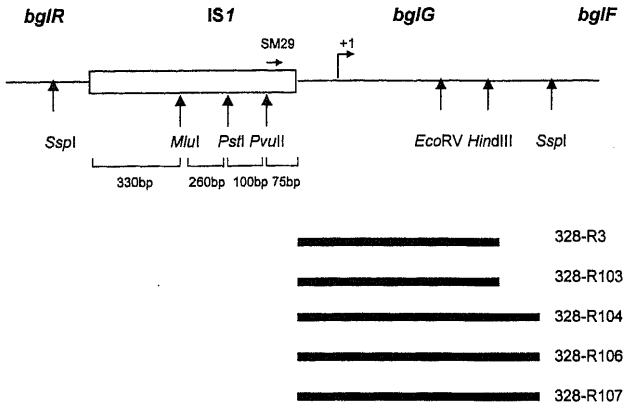

Figure 4. The extent of deletion in the revertants of AE328. The open box represents IS*1*. The thick black lines represent deletions.

recombined on to the plasmid. Restriction analysis of one such plasmid, pMN25-R3, indicated that there was no excision of the IS*1* element, but there was a ~1.0 kb deletion downstream of the insertion, which extended close to the *Ssp*I site (+965) as shown in figure 4. Sequencing of pMN25-R3 confirmed the presence of IS*1* in its entirety retained within *bglR*. Subsequent sequencing of the PCR products from revertants R103, R104, R106 and R107 confirmed the presence of the entire IS*1* in all these revertants and a deletion of genomic sequences immediately downstream to the insertion, extending into *bglG* (figure 4). These deletions are most likely promoted by the presence of the IS*1* insertion element.

Isolation of revertants under different growth conditions

The results presented above do not distinguish between Bgl$^-$ revertants that arose during exponential phase and those that arose during stationary phase. To obtain some indication regarding the growth phase during which these mutations arise, we grew cells in minimal salicin medium to stationary phase. Because no alternative carbon source was provided, any Bgl$^-$ revertant that originated during exponential phase would not multiply in the culture and their numbers will remain constant during each generation. This will result in a decrease in the frequency of Bgl$^-$ revertants detected compared to growth under nonselective conditions. Observation of a reversion frequency lower under selective conditions than under nonselective conditions would imply that the revertants originated predominantly during exponential phase. No Bgl$^-$ revertants were detected when cells were grown in a selective medium to stationary phase, suggesting that revertants originate during the exponential growth phase. When an isogenic strain carrying a mutation in the *rpoS* locus (which is known to be involved in the regulation of many stationary-phase genes) was used, the frequency of reversion in rich medium was similar to that seen in the case of the *rpoS*$^+$ strain. This result is also consistent with the idea that reversion occurs predominantly during the exponential phase of growth.

Discussion

Wild-type *E. coli* possess four different cryptic loci for utilization of β-glucoside sugars: the *bgl* operon, the *cel* operon, the *asc* operon and the *arbT* transporter (see Mukerji and Mahadevan 1997a for a review). Among these, activation by mutation is most readily seen in the case of the *bgl* operon, most of the activating mutations being due to transposition of insertion elements, which leads to enhanced transcription. Though a sophisticated mechanism exists for regulation of the operon by β-glucosides subsequent to activation, it is not clear why the genes are maintained in a silent state in wild-type cells. Activation mediated by insertion sequences provides a convenient mechanism for generating mutants that can be selected for under conditions when metabolizable β-glucosides are present in the environment. Precise excision of the element will allow reversion of the strain to the wild-type status under nonselective conditions. In that case, the cryptic state could be maintained stably by a cyclic process of activation under selective conditions and excision of the activating insertion under nonselective conditions.

The present study was undertaken specifically to test this possibility. The results presented above indicate that under laboratory conditions the frequency of precise excision is below the range that can be detected by the method employed ($<10^{-5}$). Among the 18 revertants analysed, none showed precise excision. The major limitation in detecting low frequencies of reversion to Bgl^- is the lack of a direct selection for Bgl^- mutants. Irrespective of this limitation, even though precise excision may be occurring at a low frequency, such events will be masked by the relatively high frequency of reversion due to structural-gene mutations and deletions. The latter occur at a frequency that is at least one order of magnitude higher than that of precise excisions. IS*1* is known to induce deletions in its vicinity with a high frequency (Reif and Saedler 1975). Commonly the element itself is not lost and deletions terminate precisely at one end of IS*1*. These are termed type II deletions and their formation depends on growth temperature of cells but is independent of *recA* and sequence homology. Rare type I deletions include part of the IS element along with adjacent non-IS*1* genomic sequences and can extend from 750 bp to 8500 bp (Sommer *et al.* 1981). Precise excision of IS*1* may also occur, independent of growth temperature, albeit at a low frequency of $\sim 10^{-6}$. Given the high frequency reported for type II deletions ($\sim 10^{-4}$ at 31–35°C), it is not surprising that most of the revertants we isolated have suffered deletion of the *bglR–bglG* region while retaining an intact IS*1*.

It is also interesting that the reversion events reported here occurred most likely during exponential growth. One possibility is that the results reported are laboratory artefacts, as *E. coli* in its natural habitat is possibly in a state of extended stationary phase and precise excisions occur under such conditions. This is unlikely at least for enteric strains, which probably enjoy better growth conditions. In addition, laboratory strains that carry activating mutations are stable and reversion of the activating mutation is not seen at detectable frequencies even after prolonged storage.

In the absence of true reversion under nonselective conditions, the maintenance of the cryptic state in wild-type populations is an enigma. One possible explanation is that the activated state of the operon is a case of terminal differentiation within a population and such cells will be eliminated under periodic negative selection brought about by presence of toxic substrates. However, a relatively high frequency of cells carrying deletions of the genes could result in their gradual elimination from the population after repeated cycles of alternating selection.

Another possibility for the maintenance of the operon in a cryptic state is that the regulators of the operon, which constitute a two-component response regulator, perform additional functions in the cell and the low level of expression from the silent operon is sufficient to drive these functions (Mahadevan 1997). The operon may be selected in wild-type cells primarily for these functions rather than for the catabolism of β-glucosides. This idea is consistent with the observation that in *Shigella sonnei* strains the regulatory genes are maintained and the *bglB* gene, which encodes the phospho-β-glucosidase B enzyme, is insertionally inactivated (Kharat and Mahadevan, in preparation).

Recent studies on the mechanism of silencing of the promoter suggest that the operon may be activated transiently by changes in DNA supercoiling and the concentration of H-NS protein in the cell even in absence of an activating mutation (Mukerji and Mahadevan 1997b). This brings us to the issue whether the operon is cryptic at all. If β-glucosides are considered as the primary substrates for the products of the operon, it is not clear whether any of the substrates can bring about changes in DNA supercoiling or H-NS concentrations. Resolution of these interesting questions about the function and evolution of cryptic genes will emerge only from concerted efforts at the molecular as well as the ecological level.

Acknowledgements

We thank V. Nanjundiah for comments on the manuscript and fruitful discussions. This work was supported by a grant from the Department of Biotechnology, Government of India, to S. M. S. Moorthy acknowledges the Council of Scientific and Industrial Research for a research fellowship.

References

Amster-Choder O. and Wright A. 1992 Modulation of the dimerization of transcriptional antiterminator protein by phosphorylation. *Science* **257**, 1395–1398.

DiNardo S., Voelkel K. A., Sternglanz R., Reynolds A. E. and Wright A. 1982 *Escherichia coli* DNA topoisomerase I mutants have compensatory mutations in the DNA gyrase genes. *Cell* **31**, 43–51.

Giel M., Desnoyer M. and Lopilato J. 1996 A mutation in a new gene, *bglJ*, activates the *bgl* operon in *Escherichia coli*, K-12. *Genetics* **43**, 627–635.

Hall B. G., Yokoyama S. and Calhoun D. H. 1983 Role of cryptic genes in microbial evolution. *Mol. Biol. Evol.* **1**, 109–124.

Higgins C. F., Dorman C. J., Stirling D. A., Waddel L., Broth I. R., May G. and Bremer E. 1988 Physiological role of DNA supercoiling in the osmotic regulation of gene expression in *S. typhimurium* and *E. coli*. *Cell* **52**, 569–584.

Houman F., Diaz-Torres M. R. and Wright A. 1990 Transcription antitermination in the *bgl* operon of *E. coli* is modulated by a specific RNA binding protein. *Cell* **62**, 1153–1163.

Lopilato J. and Wright A. 1990 Mechanisms of activation of the cryptic *bgl* operon of *E. coli* K-12. In *The bacterial chromosome* (ed. K. Drlica and M. Riley). American Society for Microbiology, Washington, DC.

Mahadevan S. 1997 The BglG group of antiterminators: a growing family of bacterial regulators. *J. Biosci.* **22**, 505–513.

Mahadevan S. and Wright A. 1987 A bacterial gene involved in transcription antitermination: regulation at a rho-independent terminator in the *bgl* operon of *E. coli*. *Cell* **50**, 485–494.

Mahadevan S., Reynolds A. E. and Wright A. 1987 Positive and negative regulation of the *bgl* operon of *E. coli*. *J. Bacteriol.* **169**, 2570–2578.

Mukerji M. and Mahadevan S. 1997a Cryptic genes: evolutionary puzzles. *J. Genet.* **76**, 147–159.

Mukerji M. and Mahadevan S. 1997b Characterisation of the negative elements involved in silencing the *bgl* operon of *Escherichia coli*: possible roles for DNA gyrase, H-NS, and CRP-cAMP in regulation. *Mol. Microbiol.* **24**, 617–627.

Prasad I. and Schaefler S. 1974 Regulation of the β-glucoside system in *Escherichia coli* K-12. *J. Bacteriol.* **120**, 638–650.

Reif H. J. and Saedler H. 1975 IS*1* is involved in deletion-formation in the *gal* region of *E. coli* K-12. *Mol. Gen. Genet.* **137**, 17–28.

Reynolds A. E., Felton J. and Wright A. 1981 Insertion of DNA activates the cryptic *bgl* operon in *E. coli* K-12. *Nature* **293**, 625–629.

Reynolds A. E., Mahadevan S., Le Grice S. F. J. and Wright A. 1986 Enhancement of bacterial gene expression by insertion elements or by mutation in a CAP-cAMP binding site. *J. Mol. Biol.* **191**, 85–95.

Sambrook J., Fritsch E. F. and Maniatis T. 1989 *Molecular cloning: a laboratory manual*, 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Schnetz K. 1995 Silencing of *Escherichia coli bgl* promoter by flanking sequence elements. *EMBO J.* **14**, 2545–2550.

Schnetz K. and Rak B. 1988 Regulation of the *bgl* operon of *Escherichia coli* by transcription antitermination. *EMBO J.* **7**, 3271–3277.

Schnetz K. and Rak B. 1992 A mobile enhancer of transcription in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **89**, 1244–1248.

Singh J., Mukerji M. and Mahadevan S. 1995 Transcriptional activation of the *Escherichia coli bgl* operon: negative regulation by DNA structural elements near the promoter. *Mol. Microbiol.* **17**, 1085–1092.

Sommer H., Schumacher B. and Saedler H. 1981 A new type of IS-*1* mediated deletion. *Mol. Gen. Genet.* **184**, 300–307.

Received 31 March 1998