TRANSCRIPTION ACTIVATION OF
THE *BMR* PROMOTER OF
BACILLUS SUBTILIS BY BMRR

By

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ABSTRACT

The *bmr* gene, encoding the *Bacillus subtilis* multidrug efflux protein Bmr, is positively regulated by the protein BmrR. *In vivo*, transcription of the *bmr* promoter occurs at a low, basal level in the absence of drug. When a drug substrate of Bmr such as rhodamine 6G is added to cells, transcription is enhanced approximately 18 fold from basal levels.

In vitro characterization of BmrR and RNAP binding to the *bmr* promoter showed that RNAP did not bind to linear DNA fragments containing the promoter region unless BmrR was also present. Binding of both BmrR and RNAP was enhanced approximately 2 fold when rhodamine 6G was added. Using a supercoiled template, RNAP appeared to be able to bind on its own.

Results from *in vitro* transcription assays indicated that RNAP was unable to transcribe a linear template containing the *bmr* promoter by itself. BmrR together with RNAP allowed a low level of transcription, and addition of rhodamine 6G enhanced transcription approximately 3 fold. On a supercoiled template, RNAP was capable of transcribing from the *bmr* promoter without BmrR, and BmrR itself did not enhance transcription unless rhodamine 6G was added. Thus, transcription enhancement in the presence of rhodamine 6G was likely due to enhanced binding of BmrR and RNAP.

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LIST OF ABBREVIATIONS

ATP	adenosine 5′-triphosphate
bp	base pair
BSA	bovine serum albumin
CTAB	cetyltrimethylammonium bromide
CTP	cytosine 5′-triphosphate
Da	daltons
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside-triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
GTP	guanosine 5′-triphosphate
HEPES	N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulphonic acid)
IPTG	isopropylthiogalactoside
OD	optical density
PCR	polymerase chain reaction
Phe	phenylalanine
Pro	proline
RNA	ribonucleic acid
RNAP	RNA polymerase
SDS-PAGE	sodium dodecylsulphate-polyacrylamide gel electrophoresis

TAE	Tris-acetate EDTA
TBE	Tris-borate EDTA
TE	Tris-HCl EDTA
TPP	tetraphenylphosphonium
Tris	tris(hydroxymethyl)aminomethane
UTP	uridine 5′-triphosphate

ACKNOWLEDMENT

I   INTRODUCTION

1.1   Multidrug Efflux Pumps

Antibiotic resistance is a growing problem in the clinical treatment of bacterial infections (Davies, 1994; Lewis, 1994; Salyers and Amabile-Cuevas, 1997). While rapid spread of resistance genes occurs via plasmids and conjugative transposons (Salyers et al., 1995; Amabile-Cuevas and Cardenas-Garcia, 1996; Salyers and Amabile-Cuevas, 1997), intrinsic antibiotic resistance is conferred through multidrug efflux pumps (Nikaido, 1994; Saier et al., 1998). These pumps are membrane proteins involved in the extrusion of a wide variety of toxic compounds from cells, with evidence that the drugs are removed directly from the phospholipid bilayer, thus protecting the bacteria from their ill-effects (Nikaido, 1994; Bolhuis et al., 1996; Saier et al., 1998).

Multidrug efflux pumps can be grouped into two classes: ATP-Binding Cassette (ABC) transporters (Higgins, 1992) and secondary transporters (Paulsen et al., 1996). Secondary transporters are grouped into three main families: the Small Multidrug Resistance (SMR) family (Paulsen et al., 1996), the Resistance-Nodulation-Cell Division (RND) family (Saier et al., 1994), and the Major Facilitator Superfamily (MFS) (Marger and Saier, 1993) (See Table I).

1.2   The ABC Transporters

ABC transporters are found among archaea, bacteria, and eukaryotes

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Efflux Pump Family	Average Protein size (# residues)	Number of Transmembrane Segments (TMS)	Bacterial Multidrug Transporters	Drug Substrates
ABC Transporter	1000	12 (6+6)	LmrA (*L. lactis)*	Amphiphilic cations
Major Facilitator	400	12 or 14	QacA, NorA (*S. aureus)* EmrB (*E. coli*) Bmr, Blt (*B. subtilis*)	Amphiphilic cations
SMR	100	4	Smr, QacC, Ebr *(S. aureus)* Ebr, MvrC, EmrE *(E. coli)* QacE (*K. aerogenes*)	Lipophilic cations
RND	1000	12	AcrB, YhiV (*E. coli*) MexB (*P. aeruginosa*) MtrD *(N. gonorrhoeae*)	Amphiphilic anions

Table I. Comparison of multidrug efflux pump families. Adapted from (Saier et al., 1998) and (Paulsen, Brown, and Skurray, 1996).

(Higgins, 1992). They require ATP-hydrolysis to pump toxic compounds out of cells and play an important role in transporting a large number of substances, including sugars, amino acids, ions, peptides, carbohydrates, etc. (Saier et al., 1998). These transport proteins usually exist as dimers, forming complexes with 12 transmembrane segments (TMS). They generally contain more than 1000 amino acid residues. While most ABC transporter proteins recognize and extrude only one specific substrate, there are a few examples of transporters with broad specificity, including the human multidrug resistance proteins P-glycoprotein (Gottesman and Pastan, 1993), and MRP1

(Cole et al., 1992). In bacteria, the *Lactococcus lactis* protein LmrA is the only ABC transporter that has been shown to recognize multiple amphiphilic cationic drugs (vanVeen et al., 1996).

1.3   The SMR Family

The SMR Family is a relatively new grouping of secondary transporters (Paulsen et al., 1996). Like all secondary transporters, the SMR family is dependent on the proton motive force (PMF) to drive efflux (Grinius and Goldberg, 1994). This family includes proteins such as QacC, Smr, and Ebr of *Staphylococcus aureus* (Sasatsu et al., 1989; Littlejohn et al., 1991; Grinius et al., 1992), Ebr, MvrC, and EmrE of *Echerichia coli* (Morinyo et al., 1992; Purewal, 1991), and QacE of *Klebsiella aerogenes* (Paulsen et al., 1993). All of these proteins can extrude multiple substrates, which include lipophilic cationic drugs such as ethidium bromide. Also included in the SMR family are the SugES proteins of *E. coli*, which are capable of suppressing mutations in the chaperone GroEL (Greener et al., 1993).

SMR proteins have so far only been identified in bacteria. They are characterized by being relatively small (approximately 100 amino acid residues) with only 4 membrane spanning regions (Paulsen et al., 1996). Because they are smaller than other classes of bacterial transporters, they presumably exist as oligomeric complexes (Paulsen et al., 1993).

1.4   The RND Family

Like the SMR transporters, proteins of the resistance-nodulation-cell division

family are also bacterial specific (Saier et al., 1994). They are involved in 3 main

functions: drug transport, metal ion transport, and lipooligosaccharide transport (Saier

et al., 1998). Drug transporters of this family include AcrB/F (Ma et al., 1995) and YhiV

(Ma et al., 1994) of *E. coli*, Mex B/D of *Pseudomonas aeruginosa* (Poole et al., 1996), and

MtrD of *Neisseria gonorrhoeae* (Hagman et al., 1995). CnrA (Liesegang et al., 1993),

CzcA (Nies, 1995), and NccA (Schmidt and Schlegel, 1994) of *Alcaligenes eutrophus* are

heavy metal ion transporters, and NolGHI of *Rhizobium meliloti* is a transporter of

lipooligosaccharides (Baev et al., 1991). RND proteins are large, containing

approximately 1000 amino acid residues, and they have a very characteristic topology.

There are 12 TMSs, and between the 1st and 2nd TMSs and the 7th and 8th TMSs there are

2 large hydrophilic loops which extend into the periplasm or extracytoplasmic space

(Paulsen et al., 1996; Saier et al., 1998). These loops are characteristic of this class of

proteins as they do not exist in other transporter protein classes. Mutations in the genes

for RND multidrug transporters suggest that the transporters confer resistance to

amphiphilic anionic compounds.

1.5   The Major Facilitator Superfamily

The Major Facilitator Superfamily of proteins includes up to 300 proteins which

are involved in transport of sugars, drugs, metabolites, and ions (Paulsen et al., 1996;

Marger and Saier, 1993). These transporters are ubiquitous. The bacterial multidrug

efflux pumps, which recognize cationic amphiphilic drugs, can be sub-classed into two

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groups based on protein topology. One sub-class contains proteins which have 14 TMSs, including QacA of *S. aureus* (Tennant et al., 1989) and EmrB of *E. coli* (Lomovskaya and Lewis, 1992). The other sub-class consists of proteins with 12 TMSs, such as NorA of *S. aureus* (Neyfakh, Borsch, and Kaatz, 1993), Blt (Ahmed et al., 1995), Bmr3, (Ohki and Murata, 1997) and Bmr of *Bacillus subtilis.*

Details of the mechanisms of the regulation of efflux proteins are not well known. These proteins do not all appear to be regulated in a similar fashion. For example, EmrAB of *E. coli* is negatively regulated by EmrR, a protein that represses transcription of the *emrAB* gene unless a drug substrate for EmrAB is present (Lomovskaya and Lewis, 1992). For Blt and Bmr, positive regulation is achieved by their respective regulators, BltR and BmrR, which allow transcription by binding the promoters of the *blt* and *bmr* genes when drug substrates for Blt and Bmr are present. The focus of this thesis is the detailed examination of the transcriptional regulation of the *bmr* gene.

1.6   The Bmr Protein and Its Regulation

Bmr (bacterial multidrug resistance) was identified by Alex Neyfakh's group at the University of Illinois at Chicago. The *bmr* gene was isolated from a plasmid library made from DNA of an isolate of *B. subtilis* BD170. This wild type strain had been selected on increasingly higher concentrations of rhodamine 6G, a known substrate of the human multidrug transporter P-glycoprotein, and screened for multidrug resistance (Neyfakh et al., 1991). Multidrug resistant (MDR) *Bacillus* cells have

5
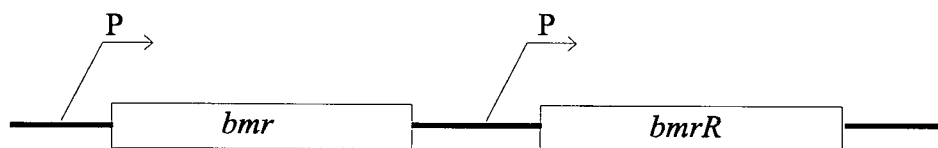
Figure 1.   Arrangement of *bmr/bmrR* gene cluster. The genes are transcribed from separate promoters.

multiple copies of the *bmr* gene, as compared to wild type cells. The cells show resistance not only to rhodamine 6G, but also to ethidium bromide, puromycin, chloramphenicol, tetraphenylphosphonium (TPP), and cetyltrimethylammonium bromide (CTAB), all substrates of P-glycoprotein. To date, it is unknown how Bmr and other multidrug efflux pumps recognize their diverse substrate compounds. However, recent studies on Bmr show that alteration of the amino acid residues Phe[143] and Phe[306] affects substrate specificity, and that Bmr may, in fact, interact directly with the drug substrates (Klyachko et al., 1997).

Directly downstream of the *bmr* gene is an open reading frame under the control of a separate promoter, which is designated *bmrR* (regulator of *bmr*) (see Figure 1). *In vivo* studies using β-galactosidase fusions to the *bmr* promoter show that *bmr* transcription is positively regulated by BmrR (Ahmed et al., 1994). BmrR activates transcription from the *bmr* promoter in the presence of drug substrates rhodamine 6G and tetraphenylphosphonium. Disruption of *bmrR* by insertion with the chloramphenicol acetyltransferase (*cat*) gene reduces drug resistance, and as shown by Northern blot analysis, prevents transcriptional activation of the *bmr* gene does not occur even in the presence of rhodamine 6G and TPP (Ahmed et al., 1994).

The two domains of BmrR have separate functions. The C-terminus of BmrR binds drug substrate, as demonstrated though equilibrium dialysis and fluorimetric analysis (Markham et al., 1996), while its N-terminus has homology to the DNA-binding N-terminus domains of the transcriptional activators MerR (Summers, 1992), SoxR (Hidalgo and Demple, 1994), TipAL (Holmes et al., 1993), and NolA (Sadowsky et al., 1991) (Figure 2). The region of homology is a proposed helix-turn-helix motif of the

regulatory proteins (Chui et al., 1996), which is implicated in DNA binding (Summers, 1992). These regulatory proteins share some unusual characteristics. All bind as dimers between the −35 and −10 regions of their target promoters, a site that usually results in transcriptional repression, not activation (Collado-Vides et al., 1991; Lanzer and Bujard, 1988). In the target promoters, the spacing between the −10 and −35 regions is also unusual because it contains 19 base pairs, as opposed to the consensus spacing of 17 base pairs. Because of the homology between BmrR and the unusual transcriptional activators, it was thought that analogies could be drawn between their known mechanisms of transcriptional activation and the possible mechanism involving BmrR.

1.7   Transcriptional Activators MerR, SoxR, TipAL, and NolA

1.7.1 MerR

MerR is involved in bacterial resistance to the heavy metal mercury in enteric bacteria. Its target promoter is the *merT* promoter, which controls expression of genes required for mercury transport (*merTP*) and reduction (*merA*) (Summers, 1992). MerR also activates expression from its own promoter, which is transcribed divergently from the *merT* promoter. In the absence of its ligand, Hg^{2+}, MerR binds to the promoter region but does not allow even a low level of transcription to occur, even though RNA polymerase (RNAP) can bind, along with MerR (O'Halloran et al., 1989). Since transcription levels in the presence of MerR, but absence of Hg^{2+}, are lower than when

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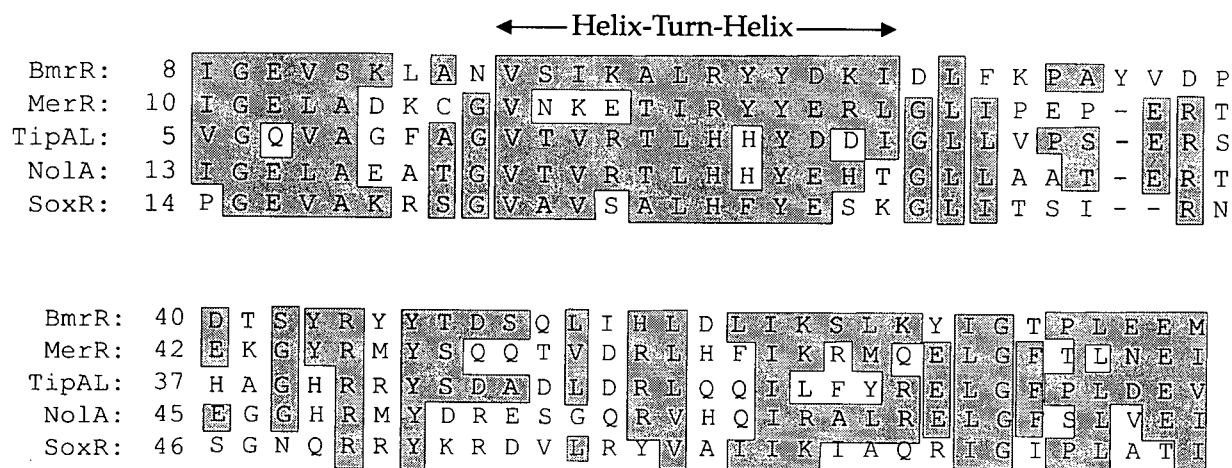
Figure 2.   Alignment of BmrR and homologous regulatory proteins. Adapted from Ahmed et al. (1994). Conserved amino acid residues are shaded.

no MerR is present in a deletion mutant (Summers, 1992), Hg^{2+} must be bound to MerR to activate transcription and without ligand, it is a transcriptional repressor. DNA footprinting using $KMnO_4$ and copper-5-phenyl-1,10-phenanthroline reveal that Hg^{2+} induces open complex formation of the *merT* promoter-MerR-RNAP complex, thus activating transcription (Frantz and O'Halloran, 1990; O'Halloran et al., 1989; Heltzel et al., 1990). Studies of the MerR-promoter complexes indicate that MerR activates transcription by untwisting the DNA at the promoter which is proposed to compensate for the irregular 19 base pair spacer region by allowing RNAP to contact the –10 and –35 elements (Parkhill et al., 1993; Ansari, Bradner, and O'Halloran, 1995).

1.7.2 SoxR

SoxR controls expression of the *soxS* promoter in *E. coli*. The SoxS protein in turn activates transcription from a regulon of stress genes which include *sodA* (superoxide dismutase gene) and *acrAB* (RND family multidrug pump gene) (Demple, 1996). SoxR contains a redox centre, made up of 2 iron and 2 sulfur atoms (2Fe-S2) that can be oxidized by superoxide or nitric oxide radicals. SoxR is capable of binding the *soxS* promoter in three forms, as apo-SoxR (SoxR lacking the 2Fe-S2 redox complex), reduced SoxR (complete with redox centre in reduced form) and oxidized SoxR (Hidalgo and Demple, 1994). RNAP can bind to the *soxS* promoter in the presence of all three forms of SoxR, but does not bind on its own. However, only the oxidized form of SoxR is capable of activating transcription from the *soxS* promoter (Ding and Demple, 1997). Unlike with MerR, a low level of transcription is observed when the apo or non-

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oxidized form of SoxR is bound to the promoter (Hidalgo and Demple, 1994), indicating that SoxR does not act as a transcriptional repressor in its inactive form. However, like MerR, activated SoxR stimulates open complex formation and distorts DNA structure as demonstrated with copper-5-phenyl-1,10-phenanthroline DNA footprint analysis (Hidalgo et al., 1995). This indicates that MerR and SoxR could have similar mechanisms of transcriptional activation.

1.7.3 TipAL

TipAL is a protein in *Streptomyces lividans* whose expression is induced by the presence of the antibiotic thiostrepton (Holmes et al., 1993). TipAL activates transcription from its autogenous promoter *tipA* and possibly other unknown promoters. Transcription from *tipA* is activated only when TipAL is bound with thiostrepton (Holmes et al., 1993; Chui et al., 1996). Like MerR and SoxR, TipAL is capable of binding the promoter without thiostrepton present, although the affinity is reduced (Holmes et al., 1993). RNAP will not bind the *tipA* promoter unless TipAL is present (Holmes, Caso, and Thompson, 1993). Transcription does not occur in the absence of thiostrepton, indicating that TipAL may act as a transcriptional repressor.

1.7.4 NolA

Little is known about the NolA protein of *Bradyrhyzobium japonicum*. It is involved in the genotype-specific nodulation of soybean plants (Sadowsky et al., 1991).

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B. japonicum forms nodules on soybean plants. Nitrogen fixation takes place within these nodules, allowing the plants to grow better. However, some soybean genotypes do not allow nodulation by *B. japonicum* serocluster 123, although they do allow nodulation by serocluster 110. A 2.3 kb region of *B. japonicum* serocluster 110 DNA was cloned and transformed into *B. japonicum* serocluster 123 cells, which allowed these cells to nodulate on soybean plants which were normally serocluster 123 restrictive. However, these cells were not capable of nitrogen fixation. The serocluster 123 cells were then conjugated to *E. coli* cells containing subcloned portions of *B. japonicum* serocluster 110 DNA and the transconjugants were used to infect soybean plants. Transconjugants able to nodulate and fix nitrogen on the serocluster 123 restrictive soybean plants were isolated. The *B. japonicum* serocluster 110 DNA region which was conjugated to the serocluster 123 cells was sequenced and named *nolA*. The NolA protein possesses the proposed helix-turn-helix motif found in the activators MerR, SoxR, TipAL, and BmrR. Its target promoter is unknown.

1.8   Thesis objectives

The objective of this thesis was to examine the mechanism of transcriptional activation of BmrR at the *bmr* promoter. Neyfakh's group showed through gel electrophoresis mobility shift assays that BmrR was capable of binding the promoter region in the presence and absence of rhodamine 6G (Ahmed et al., 1994). The homologous proteins MerR and SoxR are also capable of binding their target promoters in the presence and absence of activator. However, RNAP only binds to these target

promoters in the presence of regulator, and transcription is not activated unless the regulatory protein is in its activated form. Because of the homology between MerR, SoxR, and BmrR, the purpose of the work in this thesis was to determine the conditions required for RNAP to bind to the *bmr* promoter and for efficient *in vitro* transcription from the promoter. The questions raised include: Does RNAP require the presence of BmrR to bind and activate transcription? Does BmrR require a drug substrate to activate transcription? Does the presence of a drug substrate affect BmrR and RNAP binding to the *bmr* promoter? How does drug affect transcription activation?

2   MATERIAL AND METHODS

2.1   Bacterial Strains

E. coli strain JM109 transformed with pBmrR (E. coli expression plasmid pTrc99a from Pharmacia LKB Biotechnology Inc., containing the bmrR coding sequence between the Nco I and Bam HI sites (Ahmed et al., 1994)), was supplied by Alex Neyfakh. E. coli strain DH5α was transformed with plasmid pB (pUC19 with a 1.35 kb Eco RI fragment of chromosomal DNA from Bacillus subtilis strain BD170 selected for increased resistance to rhodamine 6G). Plasmid pB was also supplied by Alex Neyfakh.

2.2   In vitro Recombinant DNA Techniques

The bmr promoter region was recloned from plasmid pB into a pUC19 derivative containing the B. subtilis trpA transcriptional terminator (see Figure 3). Plasmid pB10 was constructed by removing the 204 bp Hind III – Bam HI spoIIG fragment from pUCIIGtrpA that was supplied by Dean Rowe-Magnus. pUCIIGtrpA is a derivative of pUC19 containing a 204 bp insert of the B. subtilis spoIIG promoter region with the B. subtilis trpA terminator inserted downstream into the Sma I site. A 336 bp PCR product was amplified from plasmid pB using primers BMRF2 and BMRR2 that flanked the promoter region of bmr (see Figure 4). BMRF2 contained a Hind III site, while BMRR2 contained a Bam HI site, allowing directional cloning of the PCR product into pUCIIGtrpA. The amplified DNA was digested with Bam HI and Hind III and ligated

Figure 3.   Cloning of the *bmr* promoter region into pUC19trpA.

EcoRI BMRR2 EcoRI
bmr

BMRF2
lac Z

pB

(pUC19:*bmr*)

ori

ampR

HindIII Bam HI

spoIIG

trpA term.

lac Z

pUCIIGtrpA

ori

ampR
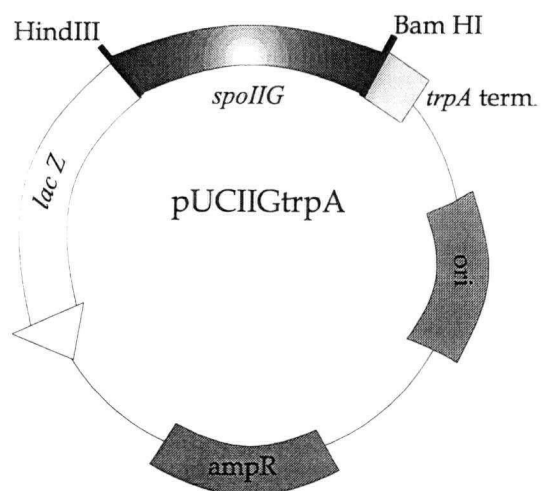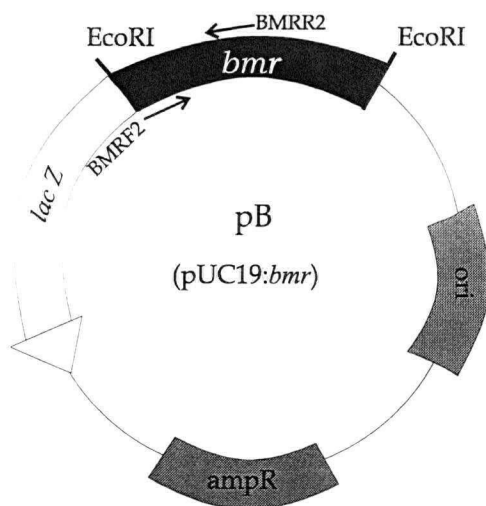
PCR *bmr* Fragment
using BMRF2/BMRR2 primers

Digest with Hind III, Bam HI
Remove *spoIIG* insert

Ligate PCR product to pUCIIGtrpA vector

HindIII Bam HI

bmr

trpA terminator

lac Z

pB10

ori

ampR

16

```
cccaagcttCCGCAGTCA TTCAGGTCCT GCCACAGCAT ATCGACATGC TTGTTCCCGC
────────────────────────────►
          BMRF2

AAATGAATAA CTGTCATAAG GCTTTAGAAA GATTTTGCAA ATCCGTTGAC TCTCCCCTAG
                                                      - 35

GAGGAGGTCT TACAGTATAA GGGATACACA CTTGAAAAAG GGGAAGTCAT ATGGAGAAGA
          -10              +1

AAAATATTAC CTTAACTATA TTATTAACCA ATTTATTTAT TGCTTTTTTG GGGATCGGGC


TTGTGATTCC AGTAACGCCG ACCATTATGA ATGAATTGCA TTTATCGGGG ACCGCGGTCG


GCTATATGGT TGCCTGCTTC GCTATTACACggatccgcg
          ◄────────────────────────────
                    BMRR2
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Figure 4.   Sequence of bmr promoter region cloned into pUC19trpA (Satola et al.,
             1992).   Position of PCR primers BMRF2 and BMRR2 are indicated by
             arrows. The –35 and –10 elements are underlined. BmrR binding site is
             highlighted in bold.

into the vector portion of pUCIIGtrpA (pUC19trpA) (Satola et al., 1992). Clones were selected on LB agar (1% Difco tryptone, 0.5% Difco yeast extract, 0.5% NaCl, 1.4% agar) containing 100 µg/ml ampicillin, to select for the ampicillin resistance gene marker on the pUC19trpA vector. Plasmid DNA was isolated from a number of clones and restricted with Bam HI and Hind III. Digests were electrophoresed through 1% TAE (40 mM Tris-acetate, 1 mM EDTA) agarose gels and positive clones were identified by having the correct-sized insert.

E. coli DH5α strains were electroporated using a "Gene pulser" apparatus from BioRad, as per manufacturer's instructions. All E. coli strains containing plasmids were maintained on LB agar containing 100 µg/ml ampicillin.

Large scale plasmid isolations were performed as per Sambrook et al. (1989), using alkali lysis and cesium chloride gradients. DNA restriction digestion and analysis of the fragments by separation on agarose gels were also performed as per Sambrook et al. (1989). DNA fragments were isolated from agarose gels using DNA isolation kits (Qiagen).

2.3 BmrR isolation

One litre of E. coli JM109 cells containing pBmrR were grown in LB medium containing 100 µg/ml ampicillin at 37°C, on a shaking platform (225 rpm). Cells were grown to O.D. $_{600}$ = 0.5 at which time IPTG was added to a final concentration of 0.5 mM. Cells were grown for an additional 2 hours and harvested by centrifugation at 7000 rpm in a Sorvall G3 rotor. Cells were resuspended in 20 mL buffer A (50 mM Tris-

HCl, pH 7.5, 1M NaCl, 5% glycerol, 2 mM β-mercaptoethanol.) Cells were lysed by French press using approximately 1200 pounds/inch² pressure. Cell lysate was cleared by centrifugation in a Sorvall SS34 rotor at 17000 rpm for 20 minutes. Solid ammonium sulfate was added to the cell lysate to 50% saturation. After stirring at room temperature for 5 minutes, precipitated proteins were recovered by centrifugation in Sorvall SS34 rotor at 1700 rpm for 20 minutes. Proteins were resuspended in buffer A and passed over a 2 ml Ω-aminooctyl agarose resin column equilibrated with buffer A. The column was washed with 5 column volumes of buffer A, until the A_{280} of the eluate from the column was less than 0.1. The column was then washed with 5 column volumes of buffer B (50 mM Tris-HCl, pH 7.5, 5% glycerol, 2 mM β-mercaptoethanol), 5 column volumes of buffer B plus 4 M urea, and protein was eluted with 2 column volumes of buffer B containing 8 M urea. The protein mixture was diluted 5 fold with dialysis buffer (20 mM Tris, pH 7.5, 100 mM NaCl) and dialyzed overnight with one 4-litre buffer change. Dialysate was stored at 4 °C. Protein concentration of the dialysate was determined using a Bradford assay (Sambrook et al., 1989) with bovine serum albumin (BSA) as standard. Protein samples from various steps of the preparation were analysed by SDS-PAGE as described by Sambrook et al. (1989).

2.4   *In vitro* Transcription Assays

Plasmid pB10, both undigested and digested with Bam HI, was used as template for *in vitro* transcriptions. Linearized template was prepared by digesting 5 µg of pB10 with 100 units of BamHI in 100 µL total volume at 37°C for 1 hour. The restriction

enzyme was extracted from the DNA using 2 phenol-chloroform extractions, as described in Sambrook et al. (1989). DNA was precipitated by adding 0.1X volume 3M sodium acetate, 3 volumes of 100% ethanol, and storage at -20°C overnight. DNA was recovered by centrifugation in an Eppendorf microfuge at 15 000 rpm for 30 minutes. The ethanol was removed and the DNA pellet was washed with 70% ethanol. The DNA was centrifuged for an additional 10 minutes and the ethanol was then removed and the pellet allowed to air dry for 10 minutes. The DNA was redissolved in TE buffer (10mM Tris-HCl, 1mM EDTA pH 7.9). DNA concentration was determined by absorption at A_{260} where 1 A_{260} = 50 ng/μL DNA.

In vitro transcription reactions were performed in 1.7 mL microcentrifuge tubes in a total volume of 10 μL. B. subtilis RNAP was a gift from Loverne Duncan. Template DNA (final concentration 3 nM) was incubated in 1X transcription buffer (10mM HEPES, pH 8.0, 10mM magnesium acetate, 1 mM DTT, 100 μg/ml acetylated BSA (Sigma), 80 mM potassium acetate) with RNAP, along with various reaction components including BmrR (up to 600 ng), and/or rhodamine 6G (10 uM), and/or initiating nucleotides ATP, CTP, and UTP (0.4 mM each), for 3 minutes at 37 °C. Initiated complexes in the reaction mix were then challenged with heparin (final concentration 10 μg/ml) and α^{32}P-GTP (3 μCi/reaction from 3μCi/mmol stock) and allowed to elongate for 5 minutes. Reactions were stopped by adding 10 μL of stop buffer (8 M urea, 0.5X TBE (45 mM Tris-borate, 1 mM EDTA), 1% xylene cyanol, 1% bromphenol blue). Transcripts were separated from free nucleotides by electrophoresis through 8% denaturing polyacrylamide gels containing 8 M urea and 0.5X TBE. All gels (including electrophoretic mobility shift assay gels) were exposed to Kodak XMR x-ray

film overnight for autoradiography. Transcripts were quantitated using a Molecular Dynamics PhosphorImager SI. The start site for the *in vitro bmr* transcript was mapped by primer extension by George Spiegelman (see Figure 5).

2.5   Electrophoretic Mobility Shift Assays

Labeled fragment for electrophoretic mobility shift (EMS) assays was generated by PCR. For labeling, 50 pmole of BMRR2 primer was suspended in 1X kinase buffer (Sambrook et al., 1989), 667 µCi γ^{32}P-ATP (ICN, 167µCi/uL), and 2 µL T4 Kinase (Gibco BRL), in a final volume of 20 µL. The reaction was incubated at 37°C for 1 hour. 50 pmoles of BMRF2 primer was then added to the mixture, along with 1 ng of pB plasmid DNA as template, 10 µL of 10X PCR buffer (200mM Tris-HCl, pH 8.4, 500 mM KCl), 10 µL of 10X dNTP (100 µM each), MgCl to 1 mM, and 1 µL of Taq polymerase, in a final reaction volume of 100 µl. The reaction was overlaid with 30 µL of mineral oil and cycled 30 times through program "BMR" (melting at 95 °C 30 seconds, annealing at 56 °C, elongation at 72 °C) using a thermocycler (MJ Research). The resulting PCR product was separated from the oligonucleotide primers and free nucleotides by electrophoresis through 5% non-denaturing polyacrylamide. The labeled PCR product was then electroeluted from the acrylamide as per Sambrook et al. (1989)

The labeled fragment was diluted such that 10 000 Cerenkov counts were used in each EMS reaction. Labeled fragment was incubated with various proteins (BmrR and/or RNAP), rhodamine 6G, and initiating nucleotides ATP, CTP, UTP (as per transcription concentrations) in 1X gel shift buffer (10 mM HEPES, pH 8.0, 10 mM

The sequence read from the gel (right side, top to bottom):

G
G
G
G
A
A
A
A
G
T
T
C
A
C
A
C
A
T
A
G ◄── Start site
G
G
A
A
T
A
T
G ⎤
A ⎟
C ⎬ -10 Site
A ⎟
T ⎟
T ⎦

Primer Extension Product ◄──
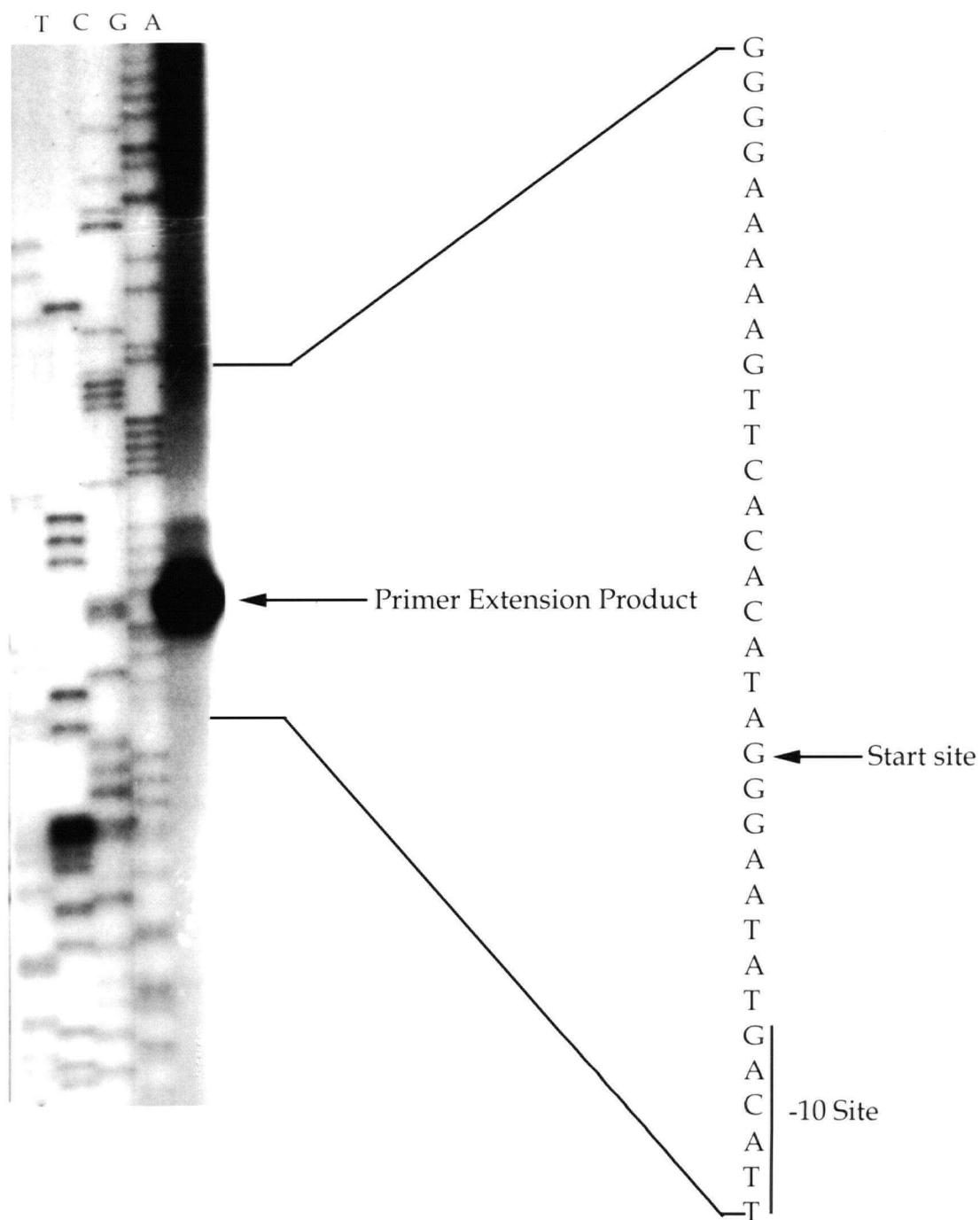
Figure 5.   Primer extension analysis of the *in vitro* bmr transcript. Lanes marked TCAG: Sequencing reactions of *bmr* promoter region using radiolabeled dNTPs. Lane marked T, sequencing reaction run with α^{32}P-dTTP. Lane marked A, sequencing reaction run with α^{32}P-dATP. Lane marked C, sequencing reaction run with α^{32}P-dCTP. Lane marked G, sequencing reaction run with α^{32}P-dGTP. Last lane: primer extension product. Start site of transcript is indicated by the arrow.

magnesium acetate, 0.1 mM DTT, 80 mM potassium acetate, 0.1 mg/ml acetylated

BSA). After incubating at 37°C for 3 minutes, reactions were stopped by adding 3 μL of

either calf thymus DNA (0.3 mg/ml in 1X Gel shift buffer containing 20% glycerol) or

heparin (0.1 mg/ml in 1X gel shift buffer containing 20% glycerol.) Complexes were

run on 4.6% non-denaturing polyacrylamide gels containing 0.83X TAE and 2% glycerol

for analysis.

3.1   Isolation of BmrR.

BmrR was isolated using a modified protocol from Alex Neyfakh, as described in Materials and Methods. Samples were taken from various steps of the isolation procedure and were separated through an 8% SDS PAGE (Figure 6). Lane 1 contains low molecular weight protein markers from BioRad. Lane 2 contains whole cell lysate before induction with IPTG. Lane 3 contains whole cell lysate after induction with IPTG. Lanes 4 to 11 contains elution fractions of BmrR from the Ω-aminooctyl agarose column eluted with buffer B containing 8M urea; BmrR is indicated by the arrow.

Elution fractions were pooled and dialyzed to remove the urea. The final concentration of BmrR was determined to be 300 ng/μL using the Bradford assay with BSA as the standard. Total recovery from 1 litre of cells was 1.2 mg of protein.

3.2   Cloning of *bmr* Promoter Region into pUCIIGtrpA.

The scheme used to clone the *bmr* promoter region into pUCIIGtrpA is presented in Figure 3. pUCIIGtrpA contained the *B. subtilis spoIIG* promoter region inserted between the Hind III and Bam HI site of pUC19. This plasmid also contained a *trpA* transcriptional terminator inserted into the SmaI site, downstream of the *spoIIG* promoter site. Thus, removal of the *spoIIG* promoter fragment and insertion of a fragment containing the *bmr* promoter region would create a construct that would be

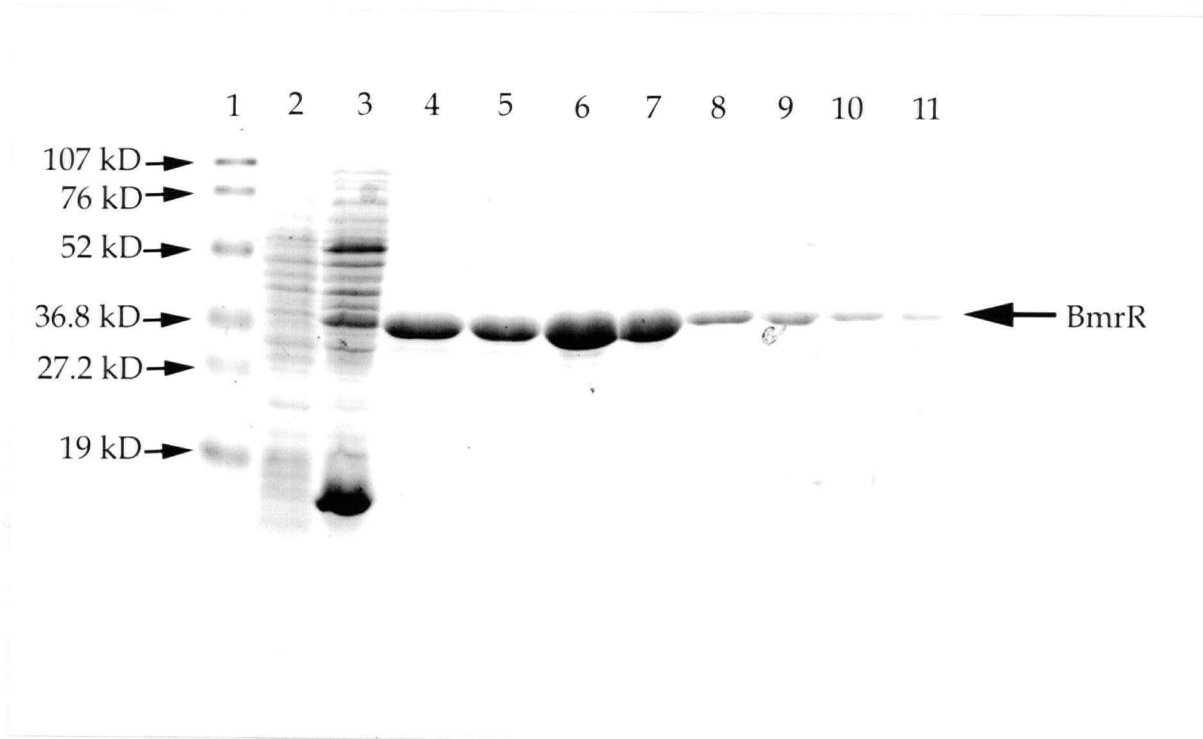Figure 6.   8% SDS-page of proteins from BmrR isolation. Approximately 10 μl of each
fraction were run in each lane. Lane 1: LMW protein markers (BioRad).
Lane 2: Cell lysate, pre-induction with IPTG. Lane 3: Cell lysate 2 hours
post-induction with IPTG. Lanes 4-11: Eluate fractions from Ω -aminooctyl
agarose resin column eluted with Buffer B containing 8 M urea. BmrR is
indicated by the arrow.

useful for *in vitro* transcription assays, as the terminator would be located downstream

of the transcriptional start site of *bmr*. The *bmr* promoter region was amplified from

plasmid pB using primers BMRF2 and BMRR2 (see Figure 7). BMRF2 was synthesized

with a *Hind* III linker, and BMRR2 was synthesized with a *Bam* HI linker. Upon

amplification, the PCR product was digested with *Hind* III and *Bam* HI to generate a

fragment 336 bp long with cohesive ends. pUCIIGtrpA was digested with *Hind* III and

Bam HI to generate the 2.7 kb pUC19trpA vector and 232 bp *spoIIG* insert. The 2

fragments were separated by electrophoresis and the vector was isolated from the

agarose. The *bmr* PCR product was ligated to the pUCIIGtrpA vector. The ligation mix

was transformed into *E. coli* DH5α. Transformants were selected on LB agar containing

100 μg/ml ampicillin. Plasmid was isolated from randomly selected clones and

digested with *Bam* HI and *Hind* III. A putative positive clone containing an insert of

approximately 300 bp was selected. The multiple cloning site of the plasmid from this

clone was sequenced and confirmed to contain the correct construct. The plasmid from

this clone was named pB10.

3.3   *In Vitro* Transcription

3.3.1   Single Round Transcription Assays Using Linear DNA Template.

Single round *in vitro* transcription assays were performed to see if the

transcriptional activation observed *in vivo* (Ahmed et al., 1994) could be mimicked *in*

vitro. The transcription assays were performed by incubating Bam HI digested

Figure 7. Results of cloning of *bmr* promoter region into pUC19trpA.

Uncut pUCIIGtrpA
pUC19trpA vector

3.0 kb—
2.0 kb—
1.6 kb—
1.0 kb—
0.6 kb—
0.2 kb—

HindIII-BamHI
spoIIG insert

336bp PCR
product
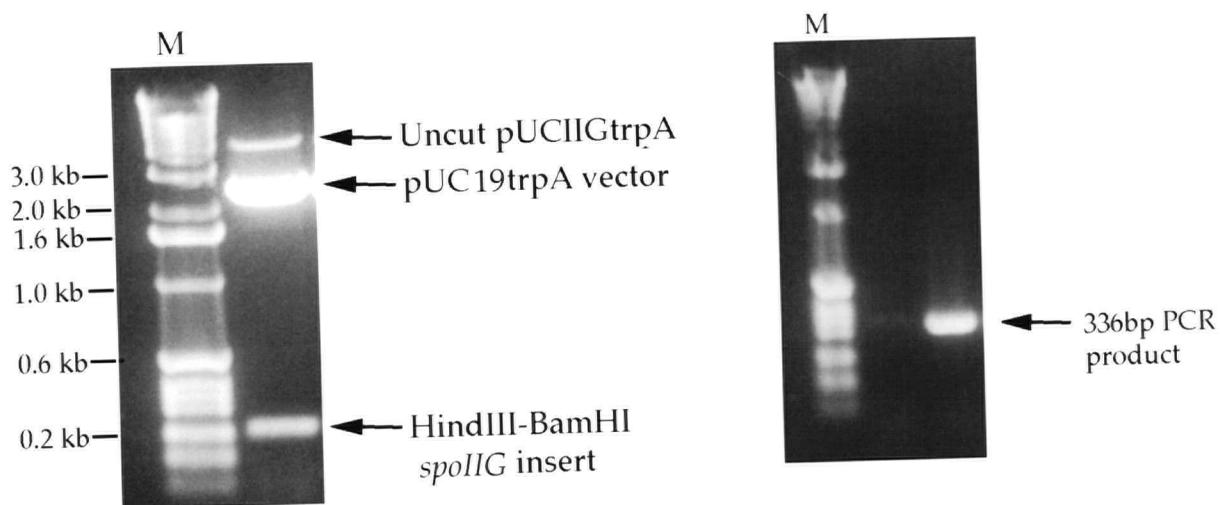
Ligate pUC19trpA vector and PCR product
Transform, select positive clones
Analyze plasmid from clones
Digest plasmid with BamHI, HindIII, screen for correct sized insert.

pUC19trpA vector

Approx. >300 bp insert
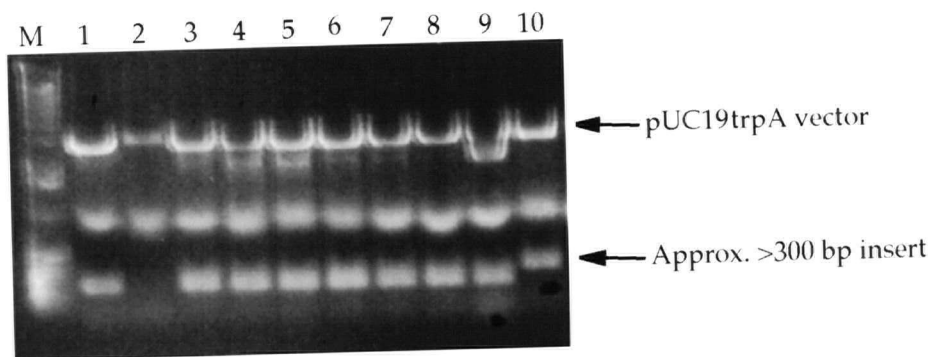
Clones pB1 to pB10.
pB10 selected for sequencing.

template DNA with various combinations of BmrR, rhodamine 6G, a drug substrate that Neyfakh used in *in vivo* transcription studies which is also a drug substrate of the mammalian multidrug transporter P-glycoprotein, initiating nucleotides ATP, CTP, and UTP, and RNAP for 3 minutes at 37°C. Reactions were then challenged with a mixture of heparin and α^{32}P-GTP. Heparin disrupts unstable complexes and prevents new complexes from forming. Only stable complexes will elongate, incorporate the radio-labeled GTP, and thus label the resulting transcripts. The products of the reactions were electrophoresed through denaturing 8% polyacrylamide gels and exposed to film to detect the transcripts.

As an initial characterization of the transcription reaction, the effects of BmrR, rhodamine 6G and initiating nucleotides on transcription were examined. Figure 8 is an autoradiograph showing the products of transcription activation of the *bmr* promoter *in vitro*. In lane 1 of the autoradiograph, no band appears indicating that no transcripts were produced from a reaction containing only template DNA and RNAP which were incubated together for 3 minutes and then challenged with heparin and nucleotides. Presumably, RNAP could not form heparin resistant initiation complexes, and thus, no transcripts were produced. When the transcription reaction contained template DNA, RNAP and 300 ng BmrR, which was then challenged with heparin and nucleotides, a low level of heparin resistant complexes were formed, as indicated by the faint band seen in lane 3. The difference between 1 and 3 indicated that BmrR stimulated formation of heparin resistant complexes. Addition of initiating nucleotides ATP, CTP, and UTP to the reaction resulted in an enhanced band (lane 4). According to the sequence of the transcript, the presence of the 3 nucleotides allowed the formation of

	1	2	3	4	5	6	7	8
pB10 Template	+	+	+	+	+	+	+	+
BmrR			+	+			+	+
ATP, CTP, UTP		+		+		+		+
Rhodamine 6G					+	+	+	+
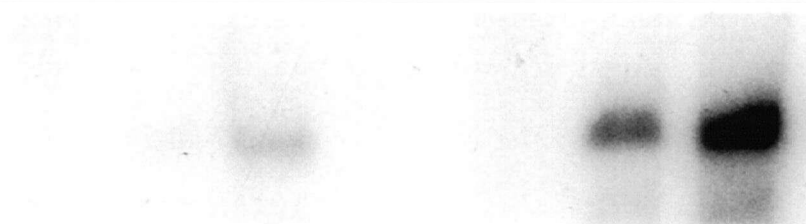
Figure 8. *In vitro* activation of transcription from the *bmr* promoter. Linearized pB10 plasmid was used as template for transcription reactions as described in Materials and Methods. Template DNA was mixed with various combinations of BmrR, RNAP, rhodamine 6G, and initiating nucleotides ATP, CTP, and UTP. The mixtures were incubated at 37°C for 3 minutes then challenged with heparin and remaining nucleotides for 5 minute elongation. Reactions were stopped with 8M urea in 0.5X TBE and reactions were run on 8% denaturing polyacrylamide to separate them from free nucleotides.

10-mers from the promoter-RNAP complex, which are more resistant to heparin challenge and thus resulted in an enhancement of the number of transcripts. The lack of a band in lane 2 indicated that initiating nucleotides could not enhance/stabilize the complexes formed by DNA and RNAP alone which would render them resistant to heparin challenge.

In lanes 5 to 8, the reactions were run with the same components as in lanes 1 to 4, with the addition of 10 μM rhodamine 6G to each reaction. No transcripts were observed in lanes 5 and 6, results similar to those seen in lanes 1 and 2, which indicated that rhodamine 6G addition alone did not stimulate transcription from the *bmr* promoter. Lanes 7 and 8 had more intense bands than those seen in lanes 3 and 4. The transcriptional activation seen in lanes 7 and 8 with rhodamine 6G indicated that the drug played a role in stabilizing or enhancing recognition of the promoter complex by RNAP. This data correlated with the *in vivo* observations that transcription activation occurs when drug substrate is present in the cells. *In vivo,* transcriptional activation of the *bmr* promoter occurs when substrate for Bmr transport is added to cells, and a low level of background transcription is observed in the absence of substrate (Ahmed et al., 1994).

The effect of BmrR and rhodamine 6G on transcription was examined further by observing transcription levels from the *bmr* promoter in the presence of varying amounts of BmrR. Transcription reactions were composed containing constant amounts of DNA template, rhodamine 6G, initiating nucleotides (ATP, CTP, and UTP), and varying amounts of BmrR. Reactions were incubated for 3 minutes at 37°C and then challenged with heparin and α^{32}P-GTP, and allowed to elongate for 5 minutes. The
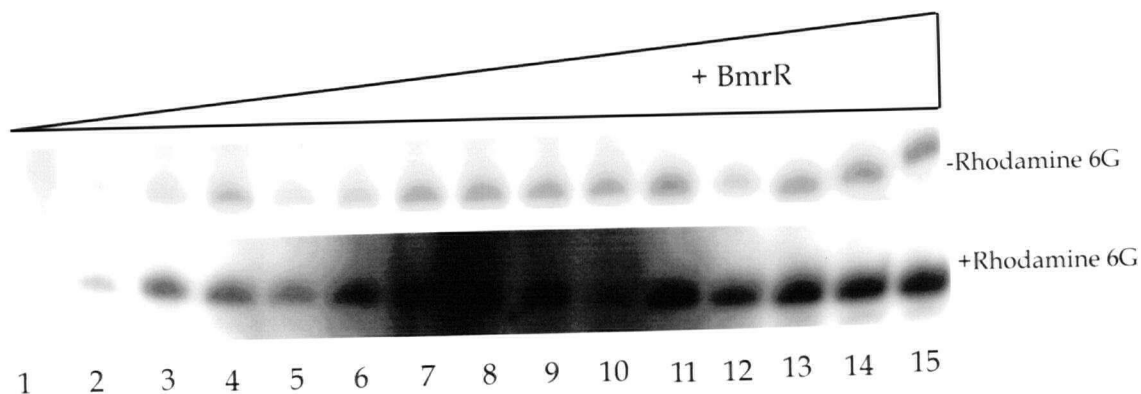
Figure 9. Effect of BmrR concentration on transcription activation. Transcription reactions were composed with constant amounts of linearized pB10 template, RNAP, initiating nucleotides ATP, CTP, and UTP, and rhodamine 6G. BmrR was added in amounts increasing from 60 ng/reaction to 600 ng/reaction. All reactions were incubated for 3 minutes at 37°C then challenged with heparin and α^{32}P-GTP. Reactions were allowed to elongate for 5 minutes then stopped with 8 M urea in 0.5X TBE. Products were electrophoresed through 8% denaturing polyacrylamide. Amount of BmrR in lanes 1 to 14 respectively: 0, 60, 120, 180, 210, 240, 255, 270, 300, 360, 420, 480, 540, 600 ng.
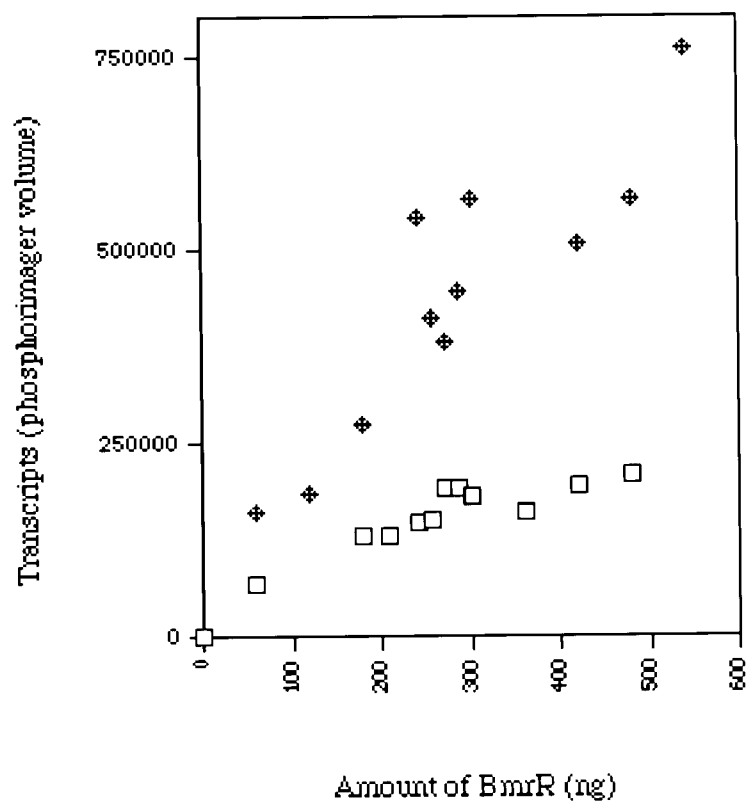
Figure 10. Quantitation of the effect of BmrR concentration on transcription
activation. Total radioactivity of the bands in Figure 9 were
quantitated by exposing gels to a phophorimager. □ - transcripts
produced in the absence of rhodamine 6G. ♦ - transcripts produced
in the presence of 10 µM rhodamine 6G.

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reaction products were electrophoresed through 8% denaturing polyacrylamide gels and autoradiographed (Figure 9). The transcripts were quantitated with a phosphorimager and plotted against BmrR input (Figure 10). In the presence of rhodamine 6G, transcription was greatly enhanced at all BmrR inputs. The graph shows that without rhodamine 6G, transcription increased slowly with BmrR input. With rhodamine 6G, transcription was enhanced approximately 3-fold above the level seen without rhodamine 6G. The increase in transcripts with BmrR was non-linear, but did not reach a maximum with the inputs tested.

3.3.2   Transcription Assays Using Supercoiled DNA Template.

In vitro transcription assays were done as described above using supercoiled plasmid DNA as template. Plasmid pB10 was used as template because the *bmr* promoter was cloned upstream of a *trpA* transcriptional terminator, which would allow termination of the transcript in the *in vitro* assays. Figure 11 is an autoradiograph of the products from transcription assay reactions composed with varying combinations of BmrR, rhodamine 6G, and initiating nucleotides. The reactions were incubated for 3 minutes at 37°C then challenged with heparin and nucleotides and allowed to elongate for 5 minutes. Reactions were stopped and transcripts separated on 8% denaturing polyacrylamide gels as described in Materials and Methods. The transcription reaction containing only RNAP and supercoiled DNA produced a high level of transcription (lane 1). This result was clearly different from the one found with linear DNA template (Figure 8), indicating that RNAP was capable of initiating transcription from the *bmr*

	1	2	3	4	5
SCpB10 Template	+	+	+	+	+
BmrR		+	+	+	+
Rhodamine 6G			+		+
ATP, CTP, UTP				+	+
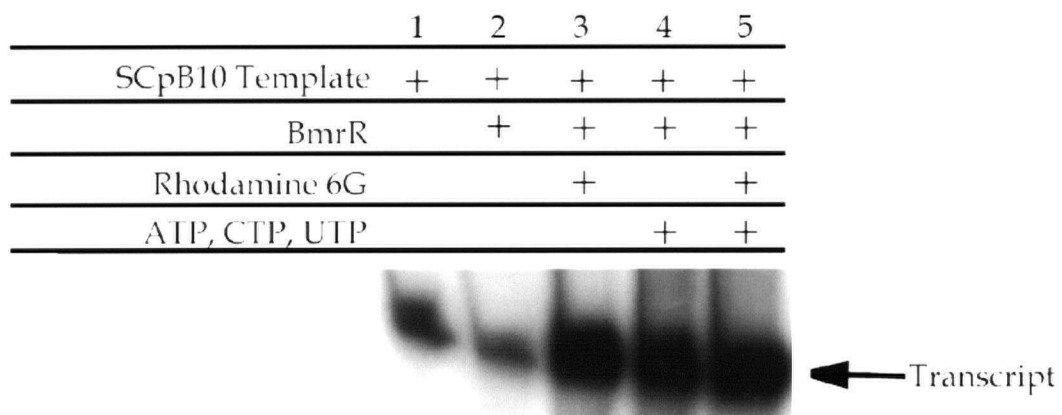
Figure 11. *In vitro* transcription analysis using supercoiled DNA template. Transcription reactions were composed as described in Materials and Methods. Supercoiled pB10 plasmid was used at template. DNA was incubated with various combinations of BmrR, rhodamine 6G, RNAP, and initiating nucleotides. Mixtures were incubated for 3 minutes at 37°C and challenged with heparin and remaining nucleotides. After 5 minute elongation, reactions were stopped with 8M urea in 0.5X TBE. Reactions were electrophoresed through 8% denaturing polyacrylamide to separate transcripts from free nucleotides.

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promoter on a supercoiled template, but not a linear template. Addition of BmrR did not significantly enhance transcription from the supercoiled template (lane 2), even though BmrR appeared to have a significant effect on transcription from the linear template. Addition of both rhodamine 6G and BmrR to the reaction in lane 3 enhanced transcription significantly over the levels seen in lane 1. The amount of transcripts produced from a reaction containing only template DNA, RNAP, BmrR and initiating nucleotides (lane 4) appeared to be slightly lower than those in lane 3, though much more intense than those in lanes 1 and 2. This indicated that initiating nucleotides enhanced transcription, likely due to stabilization of initiated complexes, as observed with the linear transcription template. However, addition of initiating nucleotides to a reaction containing both BmrR and rhodamine 6G did not enhance transcription much further (lane 5). Lanes 3 and 5 have bands with similar intensities, although the band in lane 5 appears to be slightly higher in intensity. While the results in Figure 11 were consistent with the results generated using a linear template in that the initiating nucleotides appeared to stabilize initiated complexes, the baseline transcription from the supercoiled template was markedly different from that observed using a linear template for the transcription assays.

3.4   Electrophoretic Mobility Shift Analysis

The *in vitro* transcription assays using linear template showed that BmrR and rhodamine 6G stimulated transcription initiation. However, the actual mechanism of activation was unclear. One possible mechanism of transcription enhancement was an

increase in binding of RNAP to the *bmr* promoter. To examine the effect of BmrR and rhodamine 6G on the binding of RNAP to the promoter, electrophoretic mobility shift (EMS) assays were performed. These assays were performed by incubating a labeled DNA fragment containing the *bmr* promoter region with various protein mixtures. The DNA fragment was generated by PCR amplification using an end-labeled PCR primer. The DNA-protein mixtures were incubated for 3 minutes at 37°C to allow complexes to form. The reactions were stopped with either calf-thymus DNA, a non-competitive inhibitor which does not disrupt protein-DNA complexes, or heparin, a more stringest competitive inhibitor which can disrupt unstable complexes. Reactions were then electrophoresed on native 4.7% polyacrylamide gels, which were dried and then autoradiographed. Protein-DNA complexes were seen as a band with lower mobility compared to unbound DNA.

An EMS assay was performed under the conditions of an *in vitro* transcription assay to compare the results of the transcription assays with those of the binding assay (see Figure 12). Lane 1 contained only the labeled DNA. Lane 2 contained DNA plus 300 ng of BmrR. As seen, the presence of BmrR caused the appearance of a band with lower mobility, suggesting the formation of a complex. A faint second complex was also observed. When the reaction contained only RNAP with the labeled DNA, no retarded band appeared, indicating that RNAP did not bind stably to the promoter region on its own (lane 3). When the reaction contained RNAP and BmrR together, two retarded bands were observed (lane 4). The lower band migrated at the position of the BmrR-promoter DNA complex, while the other presumably represented the RNAP-BmrR-promoter complex. The appearance of the slower migrating complex suggested
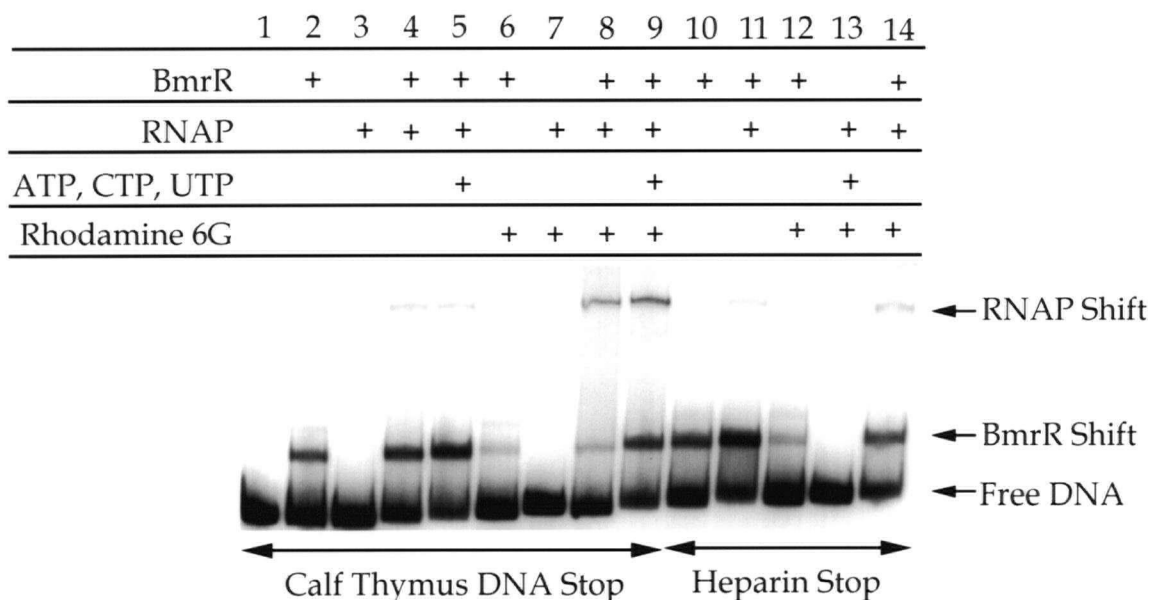
Figure 12. Electrophoretic mobility shift analysis of BmrR and RNAP binding to the *bmr* promoter. Labeled DNA fragment containing the *bmr* promoter region was incubated with various combinations of BmrR, RNAP, rhodamine 6G and initiating nucleotides ATP, CTP, and UTP. Mixtures were incubated for 3 minutes 37°C to allow complex formation. Reactions were stopped with either non-competitive calf thymus DNA or competitive heparin. Reactions were loaded onto 4.7% non-denaturing acrylamide gels to separate unbound DNA from protein-bound complexes.

that RNAP bound to the promoter only when BmrR was also present. In lane 5, the bands appeared to be the same as in lane 4, thus the addition of initiating nucleotides did not appear to enhance RNAP binding. Lanes 6 to 9 contained the same reaction components as lanes 2 to 5 with the addition of 10 μM rhodamine 6G to each reaction. In lanes 8 and 9, the upper band appeared to be enhanced, an indication that rhodamine enhanced RNAP binding. Lanes 10 to 14 contain reactions that were stopped with heparin stop buffer. Heparin is a more stringent competitor than calf thymus DNA and will normally dissociate uninitiated RNAP complexes. In lane 12, there was a band corresponding to the BmrR-DNA complex, indicating that the complex is heparin resistant. This is unusual and represents a tight association of BmrR with the DNA. RNAP binding in the presence of BmrR was partially resistant to heparin even in the absence of nucleotides. This is an unexpected finding but indicates that RNAP binding is mediated through BmrR interactions. The EMS assay results reflected the *in vitro* transcription assay data, which demonstrated that RNAP alone did not transcribe from the *bmr* promoter, but addition of BmrR allowed a low level of transcription, and addition of rhodamine 6G further enhanced transcription. In this EMS assay, RNAP was unable bind the *bmr* promoter on its own, with or without rhodamine 6G. RNAP did bind the promoter fragment with BmrR present, and this binding was enhanced when rhodamine 6G was added.

Binding of BmrR and RNAP to the promoter was examined in further detail. Figure 13 shows the results from an EMS assay of the *bmr* promoter fragment using increasing amounts of BmrR in the presence and absence of rhodamine 6G. The radioactivity in the bands was quantitated using a phosphorimager. The radioactivity

Figure 13. Effect of BmrR concentration on binding to the *bmr* promoter. Labeled fragment containing the bmr promoter region was incubated with various amounts of BmrR in the presence and absence of rhodamine 6G. Mixtures were incubated for 3 minutes at 37°C then stopped with calf thymus DNA. Reactions were loaded on to 4.7% non-denaturing polyacrylamide to separate uncomplexed DNA from protein-DNA complexes. Amount of BmrR in lanes 1 to 14 respectively: 0, 60, 120, 180, 210, 240, 255, 270, 300, 360, 420, 480, 540, 600 ng.

Figure 14. Quantitation of the BmrR concentration effect on binding to the *bmr* promoter. Total radioactivity in the DNA-BmrR complexes from Figure 13 were quantitated using a phosphorimager. Total radioactivity in each lane was also quantitated. Radioactivity in each DNA-BmrR complex was divided by total radioactivity in the lane and plotted as a percentage of total DNA shifted. □ - amount of DNA shifted in the absence of rhodamine 6G. ♦ - amount of DNA shifted in the presence of 10 μM rhodamine 6G.
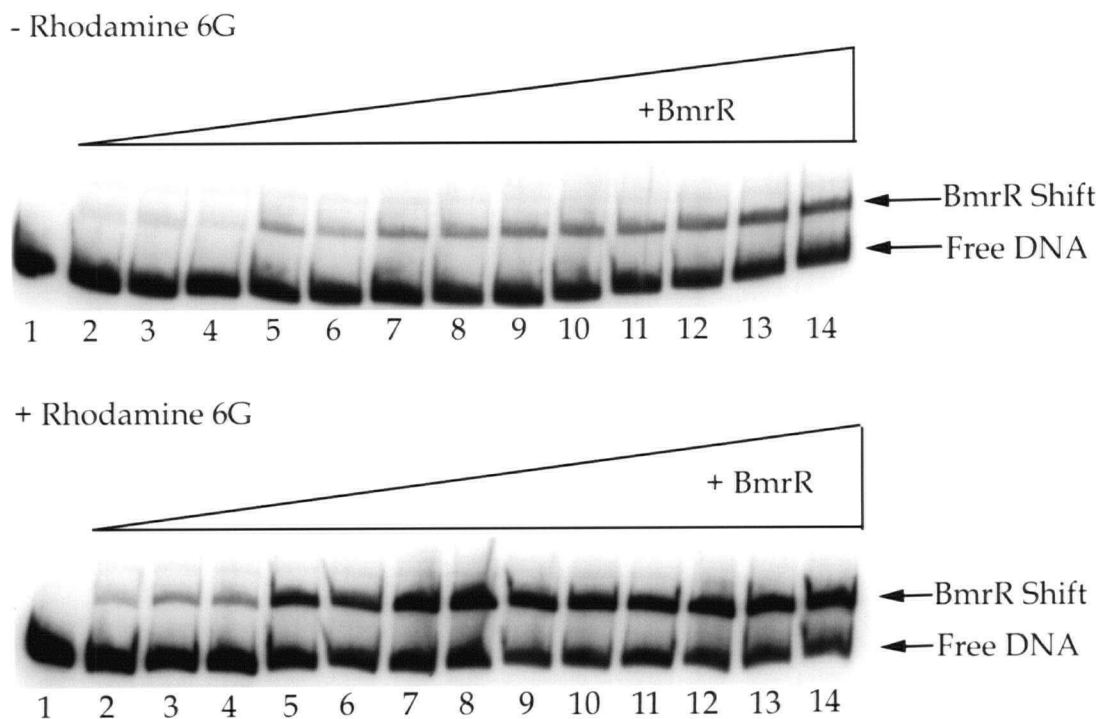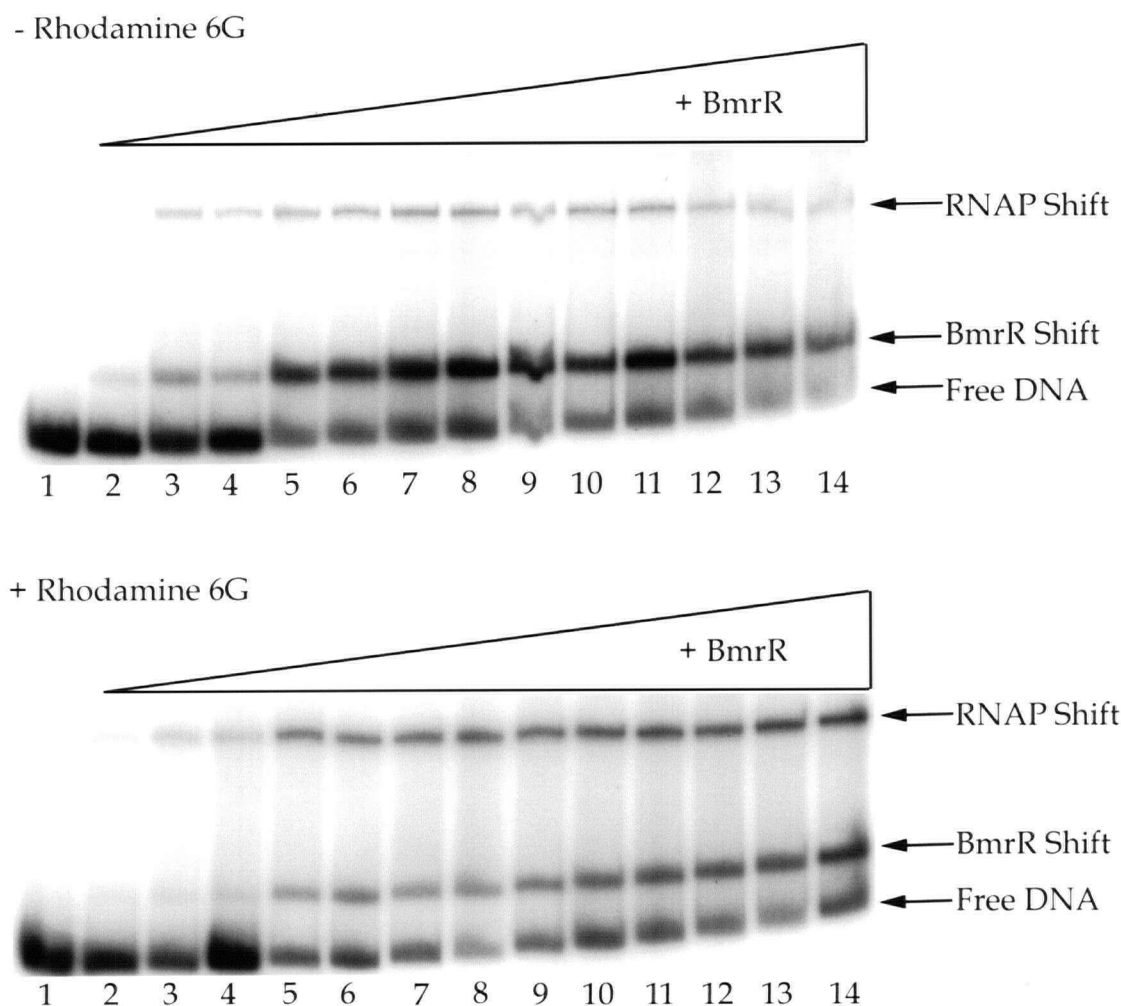
Figure 15. Effect of BmrR concentration on RNAP binding to the *bmr* promoter. Labeled fragment containing the *bmr* promoter region was incubated with constant RNAP and varying BmrR in the presence and absence of 10 μM rhodamine 6G. Reactions were incubated for 3 minutes at 37°C and stopped with calf thymus DNA. Reactions were electrophoresed through 4.7% non-denaturing polyacrymide to separate uncomplexed DNA from protein-DNA complexes. Amount of BmrR in lanes 1 to 14 respectively: 0, 60, 120, 180, 210, 240, 255, 270, 300, 360, 420, 480, 540, 600 ng.
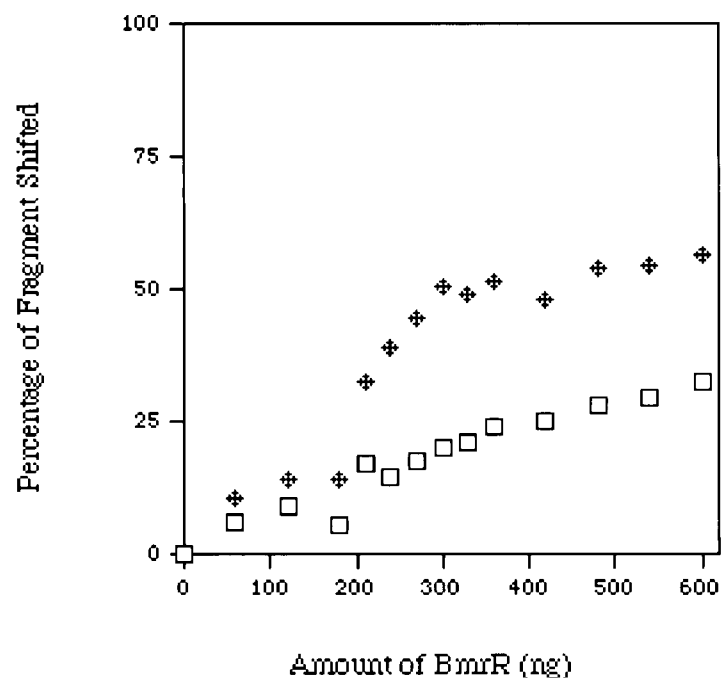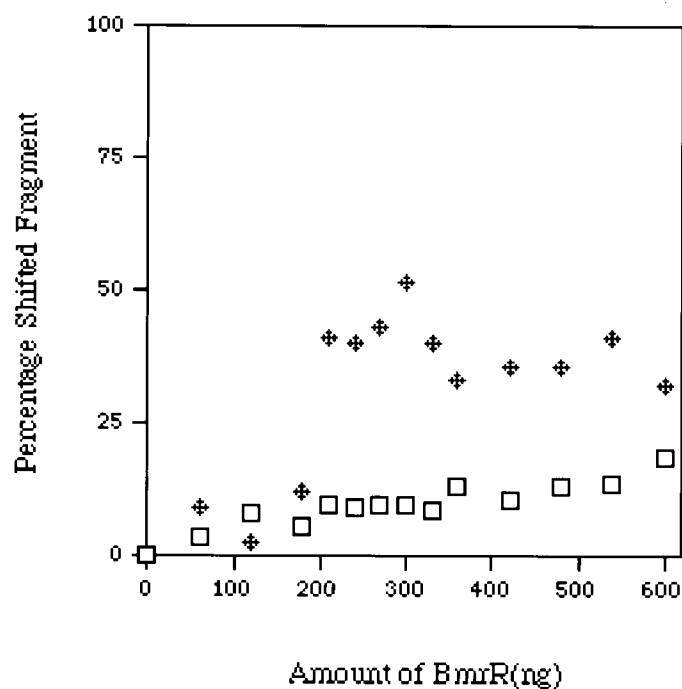
Figure 16. Quantitation of the effect of BmrR concentration on RNAP binding. Total radioactivity in the DNA-BmrR-RNAP complexes from Figure 14 were quantitated using a phosphorimager. Total radioactivity in each lane was also quantitated. Radioactivity in each DNA-BmrR-RNAP complex was divided by total radioactivity in the lane and plotted as a percentage of total DNA shifted. □ - amount of DNA shifted in the absence of rhodamine 6G. ◆ - amount of DNA shifted in the presence of 10 µM rhodamine 6G.

in each retarded band was divided by the total counts in each lane and plotted against BmrR input (see Figure 14). In the absence of rhodamine 6G, the percentage of fragment shifted increased linearly with BmrR. In the presence of rhodamine 6G, the percentage of the band shifted appeared to be a non-linear function of BmrR input. At 600 ng BmrR, the presence of rhodamine 6G increased binding by 2 fold over the level seen without rhodamine 6G. Binding of RNAP to the promoter fragment was also examined using increasing amounts of BmrR with and without rhodamine 6G, by quantitating the slower migrating fragment (Figure 15). Without rhodamine 6G, increasing BmrR resulted in a slow increase in the amount of the slower migrating complex (Figure 16). With rhodamine 6G, the increase in RNAP binding at 600 ng BmrR appeared to be 2 fold over the level seen without rhodamine 6G. The similarity in the appearance of the shapes of the RNAP and BmrR binding curves suggested that RNAP binding was dependent on BmrR binding.

3.5   EMS Analysis Using α-Subunit Mutants of RNAP

RNAP binding to the *bmr* promoter was dependent on the presence of BmrR. Because of this dependence, it is possible that protein-protein interactions exist between BmrR and one or more of the subunits of RNAP. RNAP consists of 5 subunits: 2 α subunits, a β subunit, and a β' subunit make up the core enzyme, and addition of a σ subunit confers promoter recognition specificity and completes the assembly of the RNAP holoenzyme. It has been shown that the α-subunit is required for activation at some promoters, and mutant α-subunits RNAPs have been shown to affect the activity
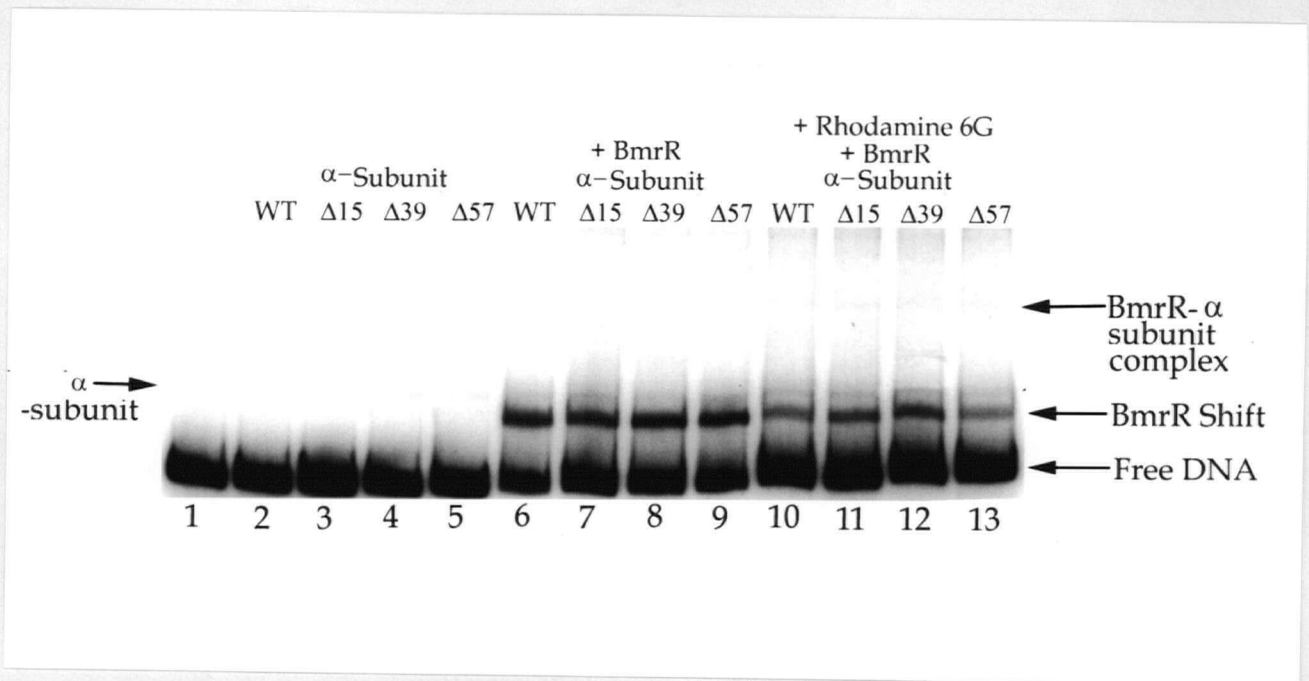
Figure 17. Electrophoretic mobility shift analysis of RNAP α-subunit mutants on the *bmr* promoter. Labeled DNA fragment containing the *bmr* promoter region was incubated with different α-subunit proteins in the presence and absence of BmrR and rhodamine 6G. Reactions were incubated at 37°C for 3 minutes then stopped with calf thymus DNA. Reactions were electrophoresed through 4.7% non-denaturing polyacrylamide to separate uncomplexed DNA from protein-DNA complexes.

of transcription activators at certain promoters (Ishihama, 1992). Thus, mutant α-subunits can be compared with wild type α-subunit to see if complexes are formed with BmrR. To test this, EMS analysis was performed using RNAP α-subunit C-terminus deletion mutants (obtained from Margarita Salas of the Centro de Biología Molecular Severo Ochoa, Universidad Autónoma, Canto Blanco, Madrid, Spain). These deletion mutants were shown by Salas' group to destabilize the binding of the *B. subtilis* phage φ29 regulatory protein p4 at the viral A3 promoter, which requires an interaction between p4 and the α-subunit of RNAP to form closed complexes (Mencia et al., 1996). The α-subunit of RNAP may interact with BmrR in a similar manner.

Three deletion mutants were obtained, one with a 15 amino acid deletion, one with a 37 amino acid deletion, and one with 59 amino acids deleted. Wild type α-subunit protein was also obtained. For the EMS reactions, approximately 100 ng of α-subunit protein was used in each reaction. The proteins were incubated with labeled DNA fragment containing the *bmr* promoter region at 37°C for 3 minutes. Reactions were stopped by addition of calf thymus DNA and were analyzed on a 4.7% non-denaturing polyacrylamide gel (see Figure 17). Lane 1 contained the labeled DNA only. Reactions in lanes 2 to 5 contained only the α-subunits and labeled *bmr* promoter fragment. All of the α-subunits appeared to bind weakly to the promoter on their own, as indicated by a faint retarded band in lanes 2 to 5. Lanes 6 to 9 contained the subunits with BmrR, and lanes 10-13 contained the subunits with BmrR in the presence of rhodamine 6G. Adding BmrR to the α-subunit protein in each reaction resulted in the appearance of 3 retarded bands (lanes 6-9). The slowest mobility band likely corresponded to the α-subunit-BmrR-complexed DNA fragment, the next faster

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mobility band corresponded to faint α-subunit-DNA complexes, and the fastest

mobility band corresponded to BmrR-DNA complexes. In lanes 10 to 13, rhodamine 6G

was added to the reactions, and there appears to be a slight enhancement of the band

representing BmrR-α-subunit-DNA complexes. The enhancement may be due to the

increased binding of BmrR to the DNA fragment that was observed previously with

rhodamine 6G. However, there were no changes in band intensities regardless of

whether wild type or mutant α-subunit protein is used in each reaction. This indicates

that the binding of the protein was independent of the mutation. Since all α-subunits

appeared identical, there was no evidence of interaction through the C-terminus

domain of the α-subunits.

4   DISCUSSION

4.1   BmrR Isolation

BmrR was isolated using a modified protocol from that described by Ahmed et al. (1994). In the isolation method described by Ahmed et al., BmrR is solubilized by addition of an oligonucleotide fragment containing the BmrR binding site from the *bmr* promoter to solubilize BmrR in solution. In the protocol used here, BmrR was simply diluted prior to the final dialysis step, which presumably prevented the protein from aggregating so that it remained in solution. The amount of active protein was not determined. There are discrepancies in the gel shift data presented here and that presented by Ahmed et al., 1994. The minimum amount of BmrR required here to produce a visible retardation of DNA was 60 ng, while Ahmed et al. only used 1 ng. This discrepancy can be explained by the differences in activity of the BmrR proteins. While protein concentration can be determined using a Bradford assay, the total amount of active protein cannot be assayed, and thus the data presented here are different from those presented by Ahmed et al.

4.2   *In vitro* Transcription Assays using Supercoiled DNA template

The results of the *in vitro* transcription assays using supercoiled DNA as template were significantly different from those generated using linear DNA as template. Using the supercoiled template, transcription from the *bmr* promoter

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occurred with RNAP alone and did not require BmrR. The presence of BmrR did not appear to activate transcription, although the presence of initiating nucleotides and/or rhodamine 6G did enhance transcription (see Figure 11). The linear template required BmrR in order to be transcribed, and rhodamine 6G enhanced transcription. Thus, by simply using a supercoiled template, transcription occurred without BmrR. Because DNA is supercoiled *in vivo*, the *in vitro* transcription assays using supercoiled template may more accurately reflect the *in vivo* situation.

Although BmrR alone did not appear to enhance transcription from the supercoiled template, the presence of rhodamine 6G and BmrR together did enhance transcription. This is still consistent with the *in vivo* transcription data where a low, basal level of *bmr* transcription occurs, and activation is seen when rhodamine 6G is added to cells (Ahmed et al., 1994). However, at issue is whether or not RNAP is capable of recognizing the *bmr* promoter on its own. The EMS assays indicated that RNAP did not bind the promoter on its own, but the DNA fragment used in these assays was also a linear fragment. If RNAP can transcribe a supercoiled template by itself, it must be able to bind to the supercoiled DNA without BmrR and/or rhodamine 6G. Thus, it seems that BmrR may play different roles in transcription initiation, depending on whether or not the DNA is supercoiled. On a linear template, BmrR was required for RNAP to bind and subsequently transcribe the *bmr* promoter, and addition of rhodamine 6G activated transcription. On a supercoiled template, RNAP bound on its own, and BmrR did not enhance transcription. Once rhodamine 6G was added, BmrR acted to enhance transcription. Rhodamine 6G alone did not affect RNAP activity. Due to the high background observed using supercoiled template for the *in*

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vitro transcription assays, as well as lack of reproducibility of some results, the interactions of RNAP and BmrR on linear template was pursued rather than the results on supercoiled template.

4.3   RNAP α-Subunit Interactions with BmrR

The EMS assay using the mutant α-subunits of RNAP did not demonstrate any obvious interactions with BmrR. The α-subunits appeared to recognize the *bmr* promoter fragment on their own, without BmrR and the α-subunit-DNA complexes appeared to be the same, regardless of whether wild type or mutant protein was used in the reactions. This indicated that either the binding was non-specific or did not involve the C-terminus of the α-subunits. However, this was not surprising, as it is known that the C-terminus domain of the RNAP α-subunit interacts with UP elements at promoters where the α-subunit enhances transcription (Ross et al., 1993) and the *bmr* promoter does not contain a recognizable UP element. It has also been reported that RNAP holoenzyme reconstituted with a C-terminus deletion mutant of α-subunit is able to transcribe from the *merT* promoter as well as wild type RNAP, indicating that MerR is a transcriptional activator that is not affected by the α-subunit (Ishihama, 1992). The *bmr* promoter and BmrR are also likely unaffected by α-subunit mutations.

4.4   Binding Enhancement of BmrR and RNAP in the Presence of Rhodamine 6G

The level of binding enhancement of both BmrR and RNAP stimulated by the presence of rhodamine 6G was determined to be 2 fold. The data from DNase I footprinting of the *bmr* promoter indicates that there is no alteration in the BmrR footprint when rhodamine 6G is present (Ahmed et al., 1994). However, recent analysis of the BmrR C-terminus (BRC) by crystallization and CD-spectroscopy reveals that rhodamine 6G may induce or stabilize the β-sheets of BmrR (Zheleznova et al., 1997). Thus, the increased binding of BmrR to the promoter in the presence of rhodamine 6G, as well as the increased binding of RNAP to BmrR-promoter complexes may be due to an altered protein structure in the C-terminus of the BmrR.

The shapes of the curves in Figures 14 and 16 suggest that cooperative binding occurs with BmrR. Neyfakh's group has shown that BmrR binds as a dimer to the *bmr* promoter (Ahmed et al., 1994). The non-linear shapes of the curves may be indicative of cooperative binding as a sharp increase in binding is observed when approximately 200 ng BmrR is added to the promoter fragment.

4.5   Analysis of Binding Enhancement and Transcriptional Activation by Rhodamine
        6G

Upon comparison of Figure 10 with Figures 14 and 16, there appeared to be a discrepancy between the transcription assay data and EMS assay data. The shapes of the curves in Figures 14 and 16 were arguably similar. Transcription enhancement and BmrR binding enhancement increase parabolically with BmrR concentration, approaching an apparent plateau. The plateau could represent saturation by BmrR or

inhibition of the transcription and binding reactions by the salt and glycerol present in the BmrR protein buffer. However, the degrees of enhancement were slightly different. While transcription was enhanced slightly more than 3 fold by rhodamine 6G (Figure 10), BmrR and RNAP binding were only enhanced 2 fold (Figures 13 and 15). This discrepancy may be partially explained by the differences between the *in vitro* transcription assay and the EMS assay. Essentially, both assays involved incubation of DNA, RNAP, BmrR, activator, nucleotides, etc., together for 3 minutes at 37°C. However, in the transcription assay, the stable complexes that were formed were immediately challenged with heparin and transcripts were allowed to elongate from the complexes. With the gel shift assays, the complexes were stopped with non-competitive calf-thymus DNA, and loaded into non-denaturing polyacrylamide gels. During the time required for the complexes to be electrophoresed into the gel matrix, they could have de-stabilized. Thus the transcriptional activation data in Figure 10 may be more representative of the effect of rhodamine 6G.

More startling, however, is the discrepancy observed between the *in vivo* transcriptional activation data and the *in vitro* data presented here. Ahmed et al. observed, using β-galactosidase fusions to the *bmr* promoter, that rhodamine 6G appeared to enhance transcription 18 fold at maximum, while TPP enhanced transcription 8 fold (Ahmed et al., 1994). The *in vitro* transcription assays here produced only a 3 fold enhancement with rhodamine 6G. Activation by TPP was not tested here.

The large gap between *in vivo* and *in vitro* transcription enhancement may indicate that *in vivo*, another positive regulatory mechanism plays a role in

transcription activation. The evidence that BmrR is required for activation *in vivo* rests on the observation that disruption of the BmrR gene results in cells with reduced drug resistance and reduced transcription of the *bmr* gene, as observed by Northern blot analysis (Ahmed et al., 1994). However, another regulatory protein could be required to enhance transcription, perhaps by binding to a site upstream of the *bmr* promoter. If this is true, neither the protein nor the upstream site were used as components of the *in vitro* transcription assays and thus, transcriptional activation levels comparable to those seen *in vivo* were not observed here. The upstream activation is not likely to involve binding of the RNAP α-subunit, as discussed earlier.

Rhodamine 6G enhanced binding of both BmrR and RNAP by 2 fold and enhanced transcription 3 fold. Thus, it is reasonable to postulate that rhodamine 6G enhanced transcription primarily through enhancement of RNAP binding. Many steps are involved in the mechanism of transcription including promoter binding by RNAP, formation of a closed complex, isomerization of the closed complex to a stable open complex, and eventually promoter escape by the RNAP and elongation of the transcript (de Haseth and Helmann, 1995; von Hippel et al., 1996). Both activated MerR and SoxR have been shown through potassium permanganate (Frantz and O'Halloran, 1990) and copper-5-phenyl-1,10-phenanthroline footprints to enhance isomerization of open complex formation (Demple, 1996). It was proposed that BmrR enhancement of open complex formation be tested through analysis of initial rates of transcription. However, extensive tests of initial *in vitro* transcription rates did not yield reproducible stimulation (data not included). A possible interpretation is that BmrR has little effect on *in vitro* isomerization rates.

The requirement for BmrR in transcriptional activation of the *bmr* promoter is likely due to the unusual spacer region between the –35 and –10 sites of the *bmr* promoter. Like the promoters recognized by MerR and SoxR, the spacer region in the *bmr* promoter is 19 base pairs, which is unusually large, as consensus promoters have spacers of 17 base pairs. RNAP is likely able to bind the –35 site but not able to contact the –10 site at the same time, and thus transcription does not occur. MerR activates transcription from the *merT* promoter by untwisting the DNA at the promoter region, thus allowing RNAP to contact the –35 and –10 sites (Summers 1992; Parkhill et al., 1993). Presumably, if the spacer region were to be mutated such that it is shortened, RNAP would be able to bind the promoter region without the activator protein. Such experiments were done with the *soxS* promoter, recognized by SoxR. The promoter was mutated such that the spacer region was shortened from 19 base pairs to 18, 17, 16, and 15 base pairs. Reducing the spacer region to 18 to 16 base pairs results in an increased basal level of transcription, and SoxR is not required for transcriptional activation (Hidalgo and Demple, 1997). With promoter spacer regions reduced to 17 base pairs or less, SoxR actually reduces RNAP binding, and essentially SoxR becomes a transcriptional repressor. This data indicates that SoxR plays an important role in altering the 3-D structure of the *soxS* promoter to allow RNAP to bind. With BmrR, DNase I footprint analysis shows that 4 hypersensitive sites appear on the *bmr* promoter when BmrR is bound (Ahmed et al., 1994), indicating an alteration in DNA configuration. Thus BmrR, like MerR and SoxR, is likely to alter the structure of the *bmr* promoter to allow RNAP to bind.

Interestingly, there are large differences in the level of transcriptional activation produced by Hg^{2+} activated MerR on the *merT* promoter and superoxide activated SoxR on the *soxS* promoter, as compared to the activation produced by rhodamine 6G activated BmrR on the *bmr* promoter. *In vivo*, activated MerR activates transcription more than 200 fold (Parkhill et al., 1993) and activated SoxR activates transcription 50 fold (Hidalgo and Demple, 1997). *In vivo*, BmrR activates transcription 18 fold at best with rhodamine 6G, and only activates transcription 8 fold with TPP. Also, while only 0.4 μM rhodamine 6G is required to achieve maximal transcription, 10 μM TPP is required for activation and produces slightly lower levels of activation (Ahmed et al., 1994), a finding that suggests that BmrR recognizes different substrates with different specificities.

Perhaps BmrR does not activate transcription to the levels achieved by MerR and SoxR on their target promoters because rhodamine 6G is not a "real" or natural activator of BmrR, but the actual activating substrate would in fact induce transcription to greater levels. This scenario is possible if the cellular function of multidrug pump proteins is to be to recognize and export drugs. Studies on the evolutionary origin of multidrug efflux pumps in bacteria show that multidrug resistance pumps have likely existed in bacteria before the advent of antibiotic usage (Saier et al., 1998). Recent research on the mammalian multidrug transporter P-glycoprotein shows that this protein may in fact have evolved to transport phospholipids (vanHelvoort et al., 1996). As well, the Bmr homologue, Blt, in *B. subtilis*, may have evolved to be a transporter or spermidine (Woolridge et al., 1997).

Recent mutations of the Pro[347] residue of Bmr by Klyachko and Neyfakh show that while alteration of this amino acid to either serine or threonine increases the efflux activity of Bmr 2 to 3 fold, the resulting mutation reduces cell viability under limiting nutrient conditions (Klyachko and Neyfakh, 1998). So, even though it is possible for Bmr to function better as an efflux pump, enhancement of drug efflux reduces viability of the cells in nutrient poor conditions, which would be common in the natural soil environment of *B. subtilis*. Thus, it has recently been hypothesized that the actual function of Bmr has to do more with survival in nutrient limiting environments than drug efflux and that drug efflux may merely be an incidental function (Klyachko and Neyfakh, 1998). Perhaps the real activator of BmrR is produced by cells under starvation conditions and induces increased transcription of the *bmr* gene. The resulting increase in the amount of Bmr protein may be required to pump out a toxic substance that would otherwise be detrimental to cell survival. If this is the case, and rhodamine 6G is not a true activator of BmrR, then the discrepancy between transcription activation levels induced by rhodamine 6G bound BmrR and those induced by activator bound MerR and SoxR can be explained. If rhodamine 6G is not a true activator, then it may not give optimal transcriptional activation *in vitro* either, which would account for the discrepancy in transcription levels observed *in vivo* and *in vitro*.

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5   CONCLUSIONS

Using a linear template to study *in vitro* transcription activation and protein-DNA interactions, RNAP was shown to be unable to bind or transcribe from the *bmr* promoter unless BmrR was also present. Addition of rhodamine 6G enhanced the binding of BmrR and RNAP to the promoter fragment by 2 fold, while transcription was activated slightly over 3 fold. Similar properties of BmrR binding and transcription activation of the *bmr* promoter were observed at the *merT* promoter and *soxS* promoters, which share similar properties with the *bmr* promoter. At these promoters, RNAP is also unable to bind unless the regulatory proteins MerR and SoxR are also bound to the promoter, and transcription is not activated unless the regulatory proteins are bound to their respective activators. While *in vivo* transcription activation levels with rhodamine 6G were observed to be approximately 18 fold, the *in vitro* results only observed a 3 fold enhancement. Also, the observation that transcription is enhanced 200 fold at the *merT* promoter and more than 50 fold at the *soxS* promoter when they are activated suggests that, if *bmr* is truly like these two promoters, rhodamine 6G may not be a true inducer of transcription activation. Thus, rhodamine 6G may not allow optimum levels of transcription *in vitro* and therefore the discrepancy between the *in vivo* and *in vitro* transcription levels observed here can be explained.

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