BECKY MARIE BUNDY
Transcriptional Regulation of the *benABCDE* Operon of *Acinetobacter* sp. Strain ADP1: BenM-Mediated Synergistic Induction in Response to Benzoate and *cis,cis*-Muconate
(Under the Direction of ELLEN L. NEIDLE)

BenM, a LysR-type transcriptional regulator of *Acinetobacter* sp. strain ADP1, controls expression of *benABCDE*, an operon involved in benzoate degradation via the β-ketoadipate pathway. *In vivo* studies suggested BenM responds to either benzoate or *cis,cis*-muconate as inducer, and both compounds together have a synergistic effect on transcription. To investigate whether the effects of these inducers result from direct interactions with BenM, we utilized a run-off *in vitro* transcription assay and DNase I footprinting. These experiments demonstrated the synergistic effects of benzoate and *cis,cis*-muconate require BenM and no additional *Acinetobacter* proteins. A C-terminally his-tagged BenM was purified by metal affinity chromatography and shown to be a stable tetramer. His-tagged BenM was as active as native BenM in the transcription assay. This assay indicated basal transcription in the absence of BenM, and a 2-fold repression of transcription in the presence of BenM without inducer. The presence of either inducer alone with BenM yielded activation. *cis,cis*-Muconate elicited a response 2-fold greater than benzoate as the sole inducer. In concert, the two inducers had a greater effect on transcription than the sum of their individual contributions. Based on DNaseI protection patterns, a model is presented in which there are three sites in the *benA* operator-promoter which BenM may bind. These sites are centered at positions -64, -43, and -12 relative to the transcriptional start site, the A residue 216 nucleotides upstream of the translational start. Without inducer, BenM binds the -64 and -12 sites, bending the intervening DNA and causing several hypersensitive sites. In the presence of either inducer, BenM no

longer protects the -12 site. When both inducers are present, BenM protects both the -64 and -43 sites. The pattern of hypersensitivity with both inducers differs from that produced with a sole inducer. Mutational analysis of the *benABCDE* promoter regulatory region suggested that Site 1 is required for cooperative binding of BenM to Sites 2 and 3, and that Site 2 is required activation of *ben* gene expression by BenM in response to either inducer alone as well as both inducers together. Therefore, both compounds in concert appear to induce conformational changes in the BenM-DNA complex that effect full induction of *benA* transcriptional activation.

INDEX WORDS: *Acinetobacter* sp. strain ADP1, BenM, CatM, LysR-type transcriptional regulator, Synergistic induction, Dual-inducer synergy, β-Ketoadipate pathway, aromatic compound degradation.

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by

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CHAPTER 1

INTRODUCTION

Purpose of This Study

These studies will focus on two transcriptional regulators, BenM and CatM. The purpose of these studies was to improve understanding of gene regulation in a soil bacterium that can degrade numerous aromatic compounds. The study of naturally occurring catabolic pathways and their regulation is an essential first step in designing bioremediation strategies for many man-made environmental pollutants. A large number of pollutants are similar in structure to compounds found in the environment. For example, halogen substituents can significantly affect biodegradation. Whereas chlorobenzoates are toxic intermediates in the slow breakdown of the pollutant polychlorinated biphenyls (PCBs), the lignin-derived aromatic compound benzoate is readily degraded by microorganisms.

Lignin, a major constituent of the cell wall of the supporting tissues of higher plants, is a complex and abundant molecule. It is the second most common polymer of plant residues (the celluloses are the most common) and makes up about 25% of the land-based biomass on earth. The decomposition of lignin is difficult and slow due to its formation by random condensation of 500-600 phenylpropane units, which can occur through both ether (C-O-C) and carbon-carbon (C-C) linkages. These linkages may occur between benzene rings, side chains, or between rings and side chains (Goodwin and Mercer, 1983). The microbial breakdown of lignin produces a vast array of aromatic

compounds similar to man-made, substituted, environmental pollutants (Alexander, 1998; Harwood and Parales, 1996; Wagner and Wolf, 1998; Goodwin and Mercer, 1983).

Lignin-derived aromatic compounds can be degraded by the bacterium *Acinetobacter* sp. strain ADP1, an aerobic, gram-type negative, non-pigmented coccobacillus found in both soil and water (Towner et al., 1991). It encodes the enzymes necessary for catabolism of many lignin-derived aromatics through the intermediate catechol (see Fig. 1.1) (Fewson, 1991; Juni, 1978). The pathway for catechol degradation is known as the β-ketoadipate pathway and includes the *benABCDE*, *catA*, and *catBCIJFD* genes (see Fig. 1.2 and 1.3) (Harwood and Parales, 1996; Neidle et al., 1986; Shanley et al., 1986; Neidle et al., 1988). The β-ketoadipate pathway of ADP1 is tightly regulated at the transcriptional level, and will be the focus of this dissertation.

Genes for aromatic compound degradation in soil microbes tend to be clustered together. The *ben* and *cat* genes of ADP1 are part of a more than 20 kbp supraoperonic cluster of genes involved in aromatic compound degradation that maps to position 2268 on the ADP1 chromosome. Map positions indicate the distance in kbp from an arbitrary origin on the ADP1 3800 kbp chromosome. A second more than 20 kbp supraoperonic cluster harboring genes for protocatechuate degradation is located at position 2567 of the ADP1 chromosome (Gralton et al., 1997; Ornston, 2001). The *antABC* operon, which I characterized earlier in my graduate studies, is responsible for the conversion of anthranilate to catechol (Bundy et al., 1998). The *ant* genes are located at map position 3500. It has not been determined whether the *ant* genes are clustered with other genes involved in aromatic compound degradation by ADP1.

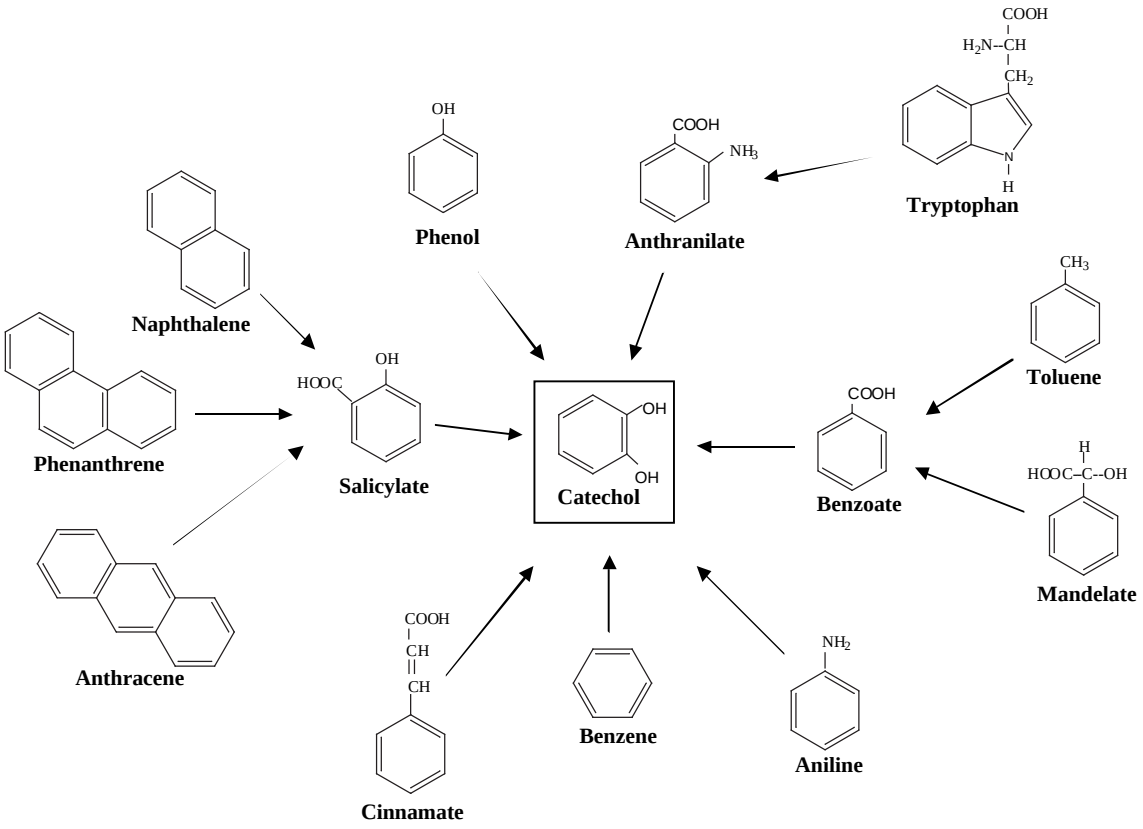
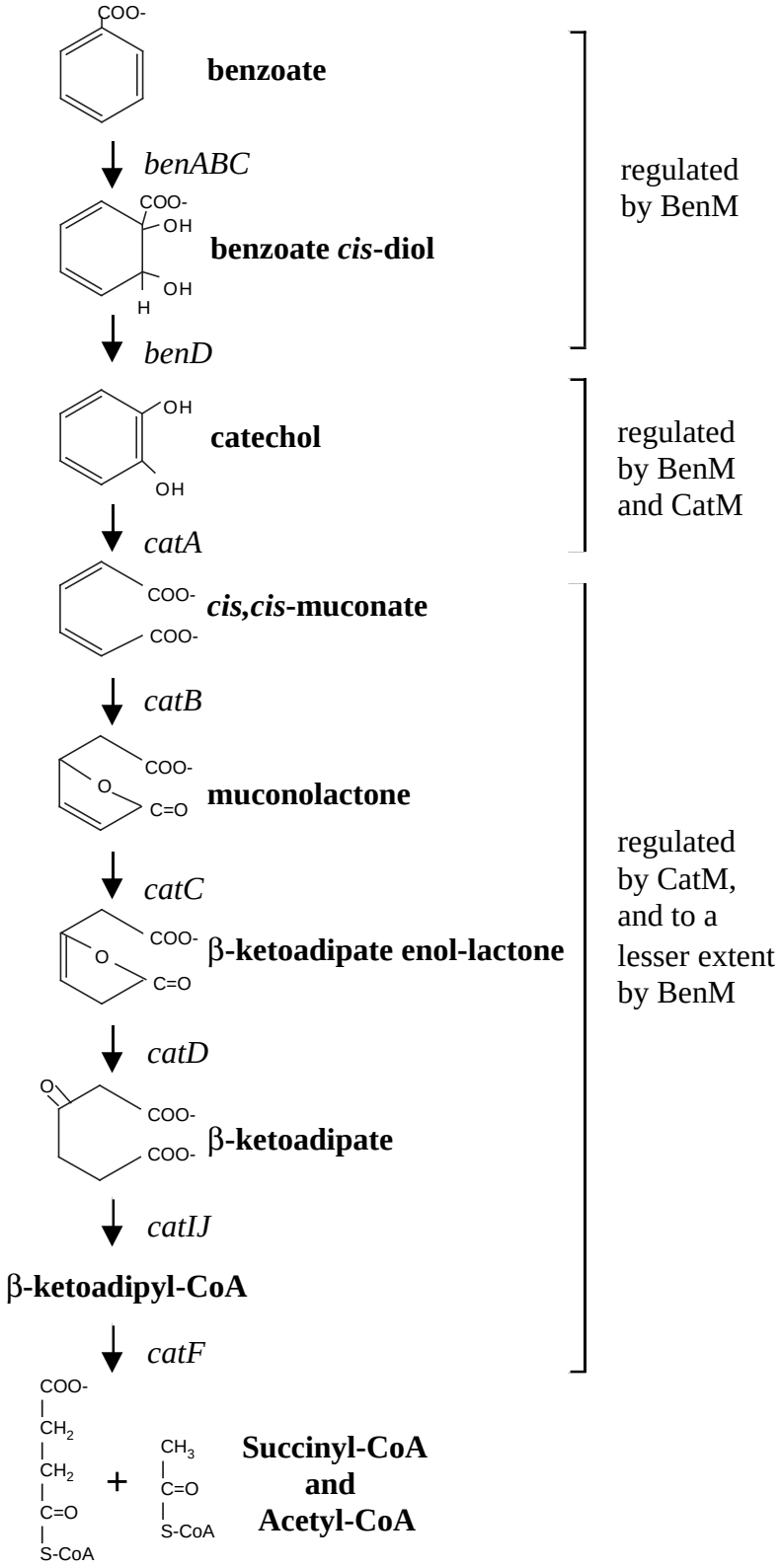


Figure 1.1. Many Lignin-Derived Aromatic Compounds Are Degraded Through the Intermediate Catechol. A few of the aromatic compounds naturally found in the environment due to the microbial break-down of plant-derived lignin are shown. These aromatics are further degraded, via the intermediate compound catechol, through the β-ketoadipate pathway (detailed in figures 1.2 and 1.3).

Figure 1.2. Genes and Intermediates of the β-Ketoadipate Pathway of ADP1. Compounds of the β-Ketoadipate pathway are displayed to the left of the figure. Corresponding genes involved in conversion of intermediates are shown between each compound in italics. Key regulatory proteins responsible for transcriptional regulation of various genes of the pathway are indicated to the right.


COO-
benzoate
benABC
COO-
OH
OH
H
benzoate cis-diol
benD
OH
OH
catechol
catA
COO-
COO-
cis,cis-muconate
catB
COO-
O
C=O
muconolactone
catC
COO-
O
C=O
β-ketoadipate enol-lactone
catD
O
COO-
COO-
β-ketoadipate
catIJ
β-ketoadipyl-CoA
catF
COO-
CH2
CH2
C=O
S-CoA
+
CH3
C=O
S-CoA
Succinyl-CoA
and
Acetyl-CoA
regulated
by BenM
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by BenM
and CatM
regulated
by CatM,
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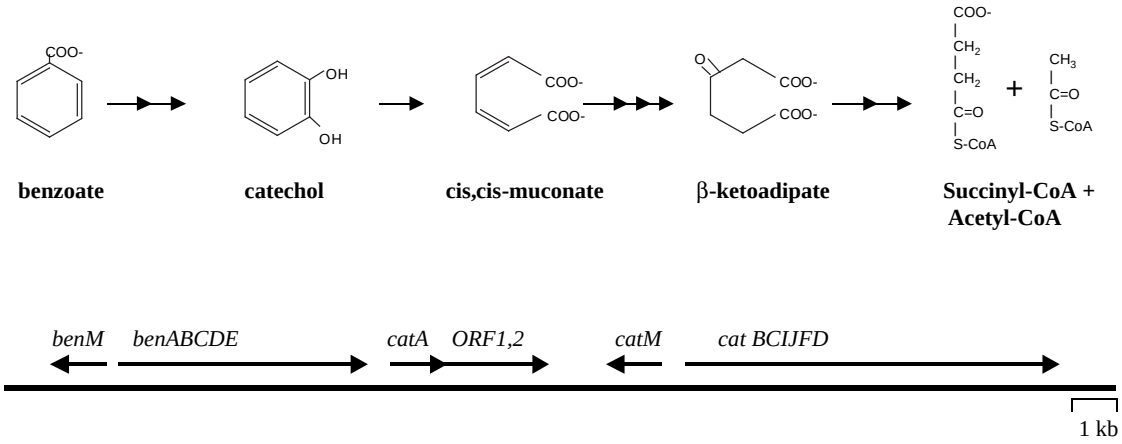


Figure 1.3. Genetic Organization of the β-Ketoadipate Pathway of ADP1. Genes involved in the degradation of benzoate via the β-Ketoadipate pathway, and their genetic organization, are displayed beneath key intermediates of the pathway.

Various possibilities exist for the clustering of aromatic compound degradation genes. These include the evolution of aromatic degradation pathways and the horizontal transfer of entire degradative pathways. In addition, this clustering may allow for complex transcriptional regulation of pathways involving toxic intermediates. The *ben* and *cat* genes of ADP1 are part of a complex transcriptional regulatory network. The genes for two LysR-Type (LTTR) transcriptional regulators, *benM* and *catM*, are located directly upstream of and divergently transcribed from the *benABCDE* and *catBCIJFD* operons, respectively (see figure 1.3) (Collier et al., 1998; Romero-Arroyo et al., 1995). BenM and CatM both activate expression of the *catA* and *catBCJIFD* operons in response to *cis,cis*-muconate (CCM). BenM is also able to activate expression of the *catA* gene in response to benzoate. BenM, but not CatM, activates expression of the *benABCDE* operon in response to either benzoate or CCM. Additionally, *in vivo* studies suggested that benzoate and CCM together allowed very high levels of *benA* expression (Collier et al., 1998). The ability of a single regulator to activate transcription synergistically in the presence of two distinct inducer compounds was novel and intriguing, but the possible involvement of additional *Acinetobacter* proteins could not be ruled out. This dissertation will deal with *in vitro* studies used to characterize this BenM-mediated inducer-synergy.

In vitro transcription assays were used to investigate *benA* gene activation and repression by the BenM and CatM proteins in response to benzoate, CCM, or both. DNase I footprinting was utilized to investigate possible conformational changes in the proteins in the presence of both inducers as compared with either inducer alone. We then took advantage of the natural transformability of ADP1 to construct a series of *benA*

promoter regulatory region mutations. *In vitro* DNase I footprinting and *in vivo* β-galactosidase assays with the promoter mutations were then utilized to help confirm a model of BenM-mediated *benA* transcriptional regulation.

The LysR-Type Transcriptional Regulators

LTTRs are one of the largest families of transcriptional regulators and are important to many diverse aspects of prokaryotic physiology. Features typical of LTTRs include (a) a highly conserved amino-terminal helix-turn-helix motif (H-T-H) that is generally ~40% identical in the amino-terminal 60 residues of all LTTRs; (b) a size of approximately 300 amino acids; and (c) the encoding gene is usually located directly upstream of and divergently transcribed from the target genes it regulates (Schell, 1993). LTTRs are generally negatively autoregulatory, and they positively regulate the expression of their target genes. In addition, LTTRs are multimeric in solution, being either dimeric or tetrameric, and they generally respond to the presence of a small coinducer molecule (Schell, 1993).

The LTTR inducer-binding regions, consisting of the centrally located residues of approximately 95-173 and 196-206 (Schell, 1993), have been determined by mutational analysis of a number of genes and by the crystallization of the C-terminal domain of both CysB and OxyR. Residue numbers are based on an alignment of NodD, NahR, AmpR, and TrpI, whose amino-terminal sequences align without gaps for the first 55 residues (Schell, 1993). Residue number 1 is the initiating methionine. The crystal structures of both CysB and OxyR align well with each other over 167 Cα carbon atoms with very little deviation (1.84 Angstroms) (Choi et al., 2001). The overall shape of the carboxy-

terminal portions of CysB and OxyR is ellipsoid, consisting of two α/β domains connected by two cross-over regions (residues 163-166 and 266-269 of CysB). Each of the two domains is a 5-stranded β-sheet with 3 interspersed α-helices. Domain I consists of residues 88-162 and 270-292 of CysB. Domain II consists of residues 166-265 of CysB. The overall structure of CysB and OxyR resembles that of the sulfate binding protein of *S. typhi* and the lysine/arginine/ornithine-binding protein of *S. typhi* (Tyrrel et al., 1997; Choi et al., 2001). It is not surprising that the CysB structure resembles that of the sulfate binding protein, since sulphide and thiosulfate act as anti-inducers of CysB-mediated transcriptional regulation. The inducer of CysB is N-acetylserine.

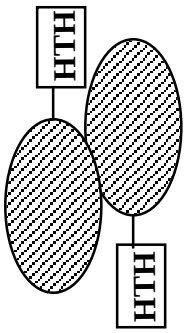
The structures of CysB and OxyR reveal a hydrophobic binding pocket, formed by residues from disparate regions of the proteins. In each case, this pocket contains a number of the residues implicated in inducer-interaction by mutational analysis. This inducer-binding pocket is formed between the two domains of the carboxyl-terminal portion of the proteins, and it is approximately 10 Angstroms deep and 6 Angstroms wide (Choi et al., 2001; Tyrrel et al., 1997). Mutational studies of LTTRs that have helped determine the inducer-binding domain include investigations of NodD (Burn et al., 1987; McIver et al., 1989), NahR (Huang and Schell, 1991; Schell et al., 1990), AmpR (Bartowsky and Normark, 1991), OxyR (Storz et al., 1990), AlsR (Renna et al., 1993), and CysB (Colyer and Kredich, 1991). Additionally, these studies show that residues in the region of 227-253, based on an alignment of NodD, AmpR, NahR, and TrpI, may also play a part in inducer response. Mutations in this C-terminal region may interfere with multimerization or LTTR-DNA interactions in response to inducer (Schell, 1993).

A typical LTTR consensus binding site contains a region of dyad symmetry with the sequence T-N_{11}-A (Schell, 1993). There are usually two binding sites containing dyad symmetry in the LTTR-regulated promoter, one of which contains a conserved T-N_{11}-A that binds strongly to the regulator in the presence or absence of inducer. A second site with some sequence similarity is often downstream from the first and on the same side of the helix (Schell, 1993). The upstream site, often termed the recognition site, binds to the LTTR and thereby increases the affinity of the regulator for the second downstream site. Binding of the LTTR to this downstream site, often termed the activation site, increases transcription in the presence of inducer (Schell, 1993). Binding of the activation site may or may not require the presence of inducer. LTTRs which bind the activation site only in the presence of inducer include CatR (Parsek et al., 1994), TrpI (Chang et al., 1990), and NahR (Schell and Sukordhaman, 1989). LTTRs that bind both the recognition and activation sites regardless of the presence of inducer include CbbR (van Keulen et al., 1998), CbnR (Ogawa et al., 1999), ClcR (Parsek et al., 1994), IlvY (Wek and Hatfield, 1986), OccR and NocR (Wang et al., 1992; von Lintig et al., 1994), NodD (Fischer and Long, 1993), and TcbR (van der Meer et al., 1991).

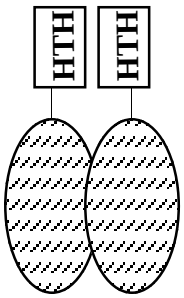
The multimeric state of LTTRs enable each dimer to contact one site of dyad symmetry, and thus each monomer contacts one half-site of the binding site. A tetrameric LTTR may therefore contact two binding sites of dyad symmetry at once, both the recognition and activation binding sites. Many LTTRs that are dimeric in solution, such as CatR and ClcR, bind to multiple sites of dyad symmetry in their respective promoter regions. Even though each individual dimer may contact only one binding site, some dimer-dimer interaction seems to occur for cooperative binding to the two

individual LTTR-binding sites (Coco et al., 1994; Parsek et al., 1992). LTTRs that are dimeric in solution, in addition to CatR and ClcR, include NodD3 (Fisher and Long, 1993), NAC (Muse and Bender, 1999), and AmpR (Bishop and Weiner, 1993). LTTRs that exist as tetramers in solution include CysB (Hryniewicz and Kredich, 1991), NahR (Schell et al., 1990), OxyR (Kullik et al., 1995), and TrpI (Chang and Crawford, 1991). Since the binding sites of LTTRs occur in tandem, the regions of protection from nucleases in footprinting assays are generally large, on the order of 50-80 bases in length. LTTRs that bind large regions of promoter DNA include CatR (Parsek et al. 1992; Chugani et al., 1997; Rothmel et al., 1991),ClcR (Coco et al., 1994; McFall et al., 1997; Parsek et al., 1994), IlvY (Rhee et al., 1998), CbnR (Ogawa et al., 1999), CysB (Hryniewicz and Kredich, 1995), and OxyR (Choi et al., 2001).

An extended footprint is seen in hydroxyl radical footprinting with CysB (Hryniewicz and Kredich, 1995). This may be due to the configuration of the CysB dimer, which places the H-T-H DNA-binding motifs of each monomer at opposite extremes of the molecule (Tyrrell et al., 1997). This is in contrast to the non-LTTR Lac repressor, which has a restricted footprinting pattern due to a configuration which places the DNA-binding domains at the same end of the dimer (see Fig. 1.4) (Lewis et al., 1996). Crystallization studies on OxyR agree well with those of CysB. Modeling of OxyR on the DNA suggested that unoxidized OxyR would occupy approximately 5 helical turns of DNA (~ 52.5 bases) with a bend in the third. Oxidized OxyR would be expected to contact 4 helical turns of DNA (Choi et al., 2001).



CysB



Lac repressor

Figure 1.4. Comparison of the Monomer-Monomer Configurations of the CysB and Lac Repressor Dimers. The orientations of the CysB and Lac Repressor monomers, within their respective dimers, is shown. The CysB dimer places the DNA-binding helix-turn-helix (HTH) motif of each monomer at opposite extremes of the molecule. This configuration accounts for the observation that CysB contacts over 40 bp of DNA at CysB-regulated promoters. The Lac Repressor places the DNA-binding HTH motifs on the same side of the molecule so that a small region of DNA is contacted at Lac Repressor-regulated promoters.

LTTR-Mediated DNA Bending

The binding of LTTRs to multiple sites in their respective promoter regulatory regions can cause bending of the intervening DNA. Evidence for this bending comes from circular permutation assays in which the binding site is placed at various positions on a DNA fragment of specific length. The regulatory protein is allowed to bind, and then the DNA-protein complex is subjected to gel mobility shift assays. If the regulator induces a bend in the DNA, the mobility of the fragment will change depending on the degree of this bend as well as the position of the bend on the fragment (Wu and Crothers, 1986). Protein-induced bends in the middle of a DNA fragment significantly slow its migration in the gel as the DNA will be more rigid over its entire length and more restricted in its movement through the gel matrix. On the other hand, if a DNA bend is introduced at the far end of a DNA fragment, the opposite end of the fragment is free to wiggle and snake its way through the gel matrix, and the complex will migrate faster (Wu and Crothers, 1986). The bending angle α is derived from the formula $\mu_M/\mu_E=\cos(\alpha/2)$ where μ_M is the mobility of the complex when the protein is bound in the middle of the DNA fragment and μ_E is the mobility of the complex when protein is bound near the end of the DNA fragment (Wu and Crothers, 1986).

Evidence for DNA bending also comes from the presence of sites hypersensitive to nuclease digestion in footprinting assays that occur in the region between the recognition and activation binding sites. These hypersensitive sites are often located at 10 bp intervals and are therefore assumed to reside on the outward curve of the DNA bend where they would be more vulnerable to nuclease digestion. Sites of hypersensitivity usually become less hypersensitive or disappear when inducer is present

to bind the LTTR. These results correlate well with circular permutation assays demonstrating that the degree of DNA bending by LTTRs is lessened in the presence of inducer. The binding of an inducer to an LTTR appears to cause a conformational change in the protein. DNA bending has been shown for a number of LTTRs, including CatR (Parsek et al., 1995), ClcR (McFall et al., 1997), OccR (Wang et al., 1992), CysB (Monroe et al., 1990), NodD (Fisher and Lang, 1993), NocR (Krensch et al., 1995), OxyR (Toledano et al., 1994), CbnR (Ogawa et al., 1999), and IlvY (Rhee et al., 1998). In most of these cases, binding of inducer to the DNA-bound LTTR results in a relaxation of the DNA bending angle, correlated with the initiation of transcription. Although the degree of promoter-DNA bending in the absence of inducer varies from system to system, the bend angle in the presence of inducer associated with the activation of transcription is near 50° in most cases studied. One exception is CatR of *P. putida*, which activates the *catABC* and *pheBA* promoters with a bend angle near 37°.

The CCM- and ChloroCCM-Binding LTTR Subclass

There are a few distinct LTTR subfamilies based on sequence homology and response to similar inducer compounds (Schell, 1993; Schlaman et al., 1992). BenM and CatM of *Acinetobacter* sp. strain ADP1 are members of a LTTR subclass that share significant similarity throughout their entire coding sequences. Members of this subclass, which respond to the inducer compound CCM or various substituted CCMs include CatR, ClcR, TcbR, TfdR, and CbnR. The inducer recognition region of BenM most closely resembles those of CatR of *Pseudomonas putida* (Rothmel et al., 1990) and CatM (Romero-Arroyo et al., 1996). These three proteins are all LTTRs that respond to CCM,

and they share approximately 60% sequence identity in their putative inducer-binding domains (residues 109 to 162 of BenM). Moreover, this region of BenM is approximately 30% identical in sequence to the central regions of the chloro-CCM responsive LTTRs ClcR (Coco et al., 1993), TcbR (Van der Meer, et al., 1991), TfdR (Leveau and Van der Meer, 1996), and CbnR (Ogawa et al., 1999). This sequence similarity, most likely reflecting the ability of BenM to respond to CCM, provides no obvious indications of interactions with benzoate, to which BenM also responds.

NahR (Schell and Poser, 1989) and PnbR (Hughes and Williams, 2001), LTTRs that respond to the substituted benzoates 2-hydroxybenzoate and p-nitrobenzoate, respectively, share less than 20% sequence identity with BenM in the putative inducer-binding domain. Recently, the LTTR responsible for activation of chlorobenzoate degradation in *Burkholderia* sp. NK8, CbeR, was identified and characterized (Francisco et al., 2001). Although BenM and CbeR may both respond to benzoate and CCM as inducers, their putative inducer-binding domains only share 44% sequence identity. BenM and CbeR are both more similar to the CCM-responsive CatR of *P. putida* than they are to each other or to the substituted benzoate-responsive LTTRs NahR and PnbR. Therefore, residues that may be important for BenM interactions with benzoate can not be inferred from sequence comparisons.

CatR- and ClcR-Mediated Transcriptional Regulation

CatR and ClcR of *P. putida* are two well-studied examples of LTTR-mediated activation by the regulators most similar to BenM. CatR regulates expression of the *catBCA* (catechol degradation), *clcABD* (2-chloro-catechol degradation), and *pheBA*

(phenol degradation) operons in response to CCM (Rothmel et al., 1990; Parsek et al., 1994; Kasak et al., 1993). ClcR regulates expression of the *clcABD* operon in response to 2-chloro-CCM (Coco et al., 1993; McFall et al., 1997). CatR and ClcR are 32.5% identical at the amino acid level and their respective promoter regulatory regions at *catBCA* and *clcABD* are 50% identical (Coco et al., 1993). Though CatR is able to bind and activate transcription of the *catBCA*, *pheBA*, and *clcABD* operons, ClcR only activates expression of the *clcABD* operon (Parsek et al., 1994). Though CatR and ClcR are not known to respond synergistically to multiple inducer compounds, the CatR/ClcR-mediated model of transcriptional activation shares some key features with our BenM/CatM-mediated model of *benA* expression.

DNase I footprinting experiments demonstrated that CatR protects the recognition binding site, bases -79 to -54 relative to the *catBCA* transcriptional start site, in the absence of the inducer CCM. In the presence of CCM, CatR additionally protects bases -47 to -33 corresponding to the activation binding site (Parsek et al., 1992; Rothmel et al., 1991). When CatR is bound to the recognition site in the absence of inducer it bends the *catBCA* promoter region 59°. This DNA-bend accounts for sites hypersensitive to DNase I cleavage observed at -48 and -49 in the absence of inducer. The degree of promoter-DNA bending is reduced to 37° in the presence of CCM. Binding to the activation site correlated with the disappearance of the DNase I hypersensitive sites (Parsek et al., 1992).

CatR also regulates expression of the *pheBA* operon in response to CCM. In the absence of CCM, CatR protects the recognition binding site, from -52 to -77 with respect to the *pheBA* transcriptional start site, and bends the DNA to an angle of 34°. When

CCM is present, CatR additionally protects the activation site, from -30 to -45, and the promoter DNA is bent 38°. Although the degree of DNA-bending does not significantly change upon interaction with inducer, the degree of bending observed with CatR at *pheBA* closely resembles that observed with CatR at *catBCA* in the presence of CCM. In fact, the footprints of CatR at both the *catBCA* and *pheBA* promoters are nearly identical, leading to the proposal that the configuration for CatR activation is conserved (Parsek et al., 1995).

In contrast to CatR, which only binds the activation site upon interaction with inducer, DNase I footprinting of ClcR at the *clcABD* promoter demonstrated that ClcR binds both the recognition and activation sites regardless of the presence of inducer. In the absence of 2-chloro-CCM, ClcR protects bases -79 to -53 and -37 to -28, with respect to the *clcABD* transcriptional start site. In addition, sites hypersensitive to DNase I cleavage in the absence of inducer are observed at -52, -51, and -42. When 2-chloroCCM is present, ClcR-binding of the activation site shifts slightly from protecting bases -37 to -28 to protecting bases -41 to -32. This footprint of ClcR at the *clcABD* promoter in the presence of inducer closely resembles that of CatR at the *catBCA* promoter in the presence of inducer (Coco et al., 1993; McFall et al., 1997). In contrast to CatR-mediated *catBCA* activation, ClcR bends the *clcABD* promoter regulatory region 71° in the absence of inducer and 55° in the presence of inducer. This reduction in DNA-bending angle correlates with the loss of the DNase I hypersensitive sites in the footprinting assays when inducer is absent.

DNase I footprinting of CatR at *clcABD* demonstrated that CatR protects both the recognition and activation sites regardless of the presence of CCM. This is different from

its protection at the *catBCA* promoter where the activation site is only bound in the presence of CCM. In fact, CatR was shown to activate expression of the *clcABD* operon even in the absence of inducer, in a concentration-dependent manner, though addition of CCM increased *clcABD* expression 2-3 fold (McFall et al., 1997). The footprint of CatR at *clcABD*, regardless of the presence of inducer, is identical to that of ClcR at *clcABD* in the presence of 2-chloroCCM. In addition, CatR bends the *clcABD* promoter regulatory region to 54° regardless of the presence of CCM. The degree of DNA-bending induced by CatR with or without inducer is virtually identical to the angle ClcR bends the *clcABD* promoter in the presence of inducer, 55° (McFall et al., 1997). It was therefore proposed that the mechanism of *clcABD* transcriptional activation is conserved between CatR and ClcR. Though the degree of DNA bending required for transcriptional activation is different between the *catBCA*/*pheBA* and *clcABD* promoter regulatory regions, 37-38° and 54-55° respectively, the footprinting patterns of CatR at the *catBCA*, *pheBA*, and *clcABD* promoters in the presence of inducer, as well as the footprint of ClcR at the *clcABD* promoter in the presence of inducer, are all very similar. The regulatory protein is bound to a recognition binding site around the –50 to –70 region, with respect to the transcriptional start site, as well as an activation binding site around –30 to –40 (McFall et al., 1998).

Recently, a third CatR binding site was discovered in the *catB*, *pheB*, and *clcA* structural genes (Chugani et al., 1998). This additional LTTR-binding site has very low affinity for CatR and was named the internal binding site (IBS). DNase I footprinting of CatR at the *catB* IBS demonstrated CatR protection of bases +162 to +196 (Chugani et al., 1998). Binding of CatR to the IBS was increased in the presence of both the

recognition and activation sites, demonstrating cooperative binding of CatR. Binding to the IBS then creates a DNA-loop. Addition of 11 bases of DNA (a complete helical turn) between the activation site and IBS did not affect *catBCA* expression. Addition of 5-6 bases resulted in expression levels seen in an IBS mutant strain. These levels indicated that binding of CatR to the IBS, and to the recognition and activation sites, results in 3-4 fold repression of the *catBCA* operon. The IBS is therefore a *cis*-acting repressor element that helps keep production of the toxic intermediate CCM in check (McFall et al., 1998).

Many LTTRs are Class I Type Transcriptional Regulators

Binding of both CatR and ClcR to their respective promoter regulatory regions just upstream of, and overlapping, the –35 RNA Polymerase (RNAP) promoter region enables these LTTRs to function as Class I type transcriptional activators (Chugani et al., 1997; McFall et al., 1997). Class I transcriptional activators require contacts with the carboxy-terminal portion of the α-subunit (α-CTD) of RNAP for activation of transcription (Ishihama, 1993). Since the α-CTD of RNAP is loosely tethered to the upstream-facing portion of RNAP, binding of regulatory proteins in the vicinity of the –35 RNAP promoter regulatory region places them in perfect position for interacting with the α-CTD. Studies with RNAP α-CTD mutants and deletions have demonstrated that a number of LTTRs are Class I transcriptional regulators. These include OxyR (Tao et al., 1993), TrpI (Gussin et al., 1992), CysB (Shi and Bennett, 1994), CatR (Chugani et al., 1997), and ClcR (McFall et al., 1997). ClcR also demonstrates signs of being a Class II transcriptional activator, one which contacts the σ-subunit of RNAP holoenzyme (Ishihama, 1993). Studies with RNAP α-CTD mutants and deletions did not completely

eliminate *clcABD* expression in *in vitro* transcription studies (McFall et al., 1997). ClcR may therefore be making productive contacts with the σ-subunit of RNAP as well as the α-CTD for transcription initiation of the *clcABD* operon. The interactions between BenM, CatM, and RNAP in *Acinetobacter* sp. strain ADP1 remain to be investigated.

The Natural Transformation System of *Acinetobacter* sp. Strain ADP1

Acinetobacter sp. strain ADP1, previously known as strain BD413, is particularly well suited to studies of transcriptional regulation due to its competency for natural transformation. The natural transformation system of ADP1 enables the construction of chromosomal mutations, knock-outs, and insertions with relative ease. ADP1 was isolated through UV-induced mutagenesis of the environmental isolate *Acinetobacter* sp. strain BD4. Unencapsulated mutants were identified on the basis of morphology. One microencapsulated mutant which retained a high level of competency for natural transformation, later known as *Acinetobacter* sp. strain ADP1, was selected for further study because it did not "clump" when grown in liquid media (Juni and Janik, 1969).

The natural transformation system of *Acinetobacter* sp. strain ADP1 has recently been investigated. There is a high competency for transformation induced at the transition from the lag to the exponential phase of growth, with a gradual decline thereafter. DNA uptake requires energy and is dependent on the presence of divalent cations such as Mg^{2+}, Ca^{2+}, and Mn^{2+} (Palmen et al., 1993; Cruze et al., 1979). ADP1 does not discriminate between heterologous and homologous DNA, and uptake is in a single-stranded form with integration into the chromosome via a RecA-dependent mechanism (Porstendorfer et. al., 1997; Gregg-Jolly and Ornston, 1994).

The Averhoff group identified 5 genes of *Acinetobacter* sp. strain ADP1 required for natural transformation using insertional mutagenesis and transposon shuttle mutagenesis. Three of these genes, *comP*, *comE*, and *comF* were all shown to be significantly similar to the type IV pilins and are required for natural transformation, but not essential for pilus biogenesis or function (Porstendorfer et. al., 1997; Busch et. al., 1999). The fourth gene, *comC*, is similar to proteins involved in the assembly of type IV pili, and was found to be required for transformation of ADP1, but not essential to pilus formation (Link et. al., 1998). *ComC* and *comP* were additionally shown to be required for DNA binding and uptake (Link et al., 1998; Porstendorfer et al., 1997).

Type IV pili are filamentous appendages found on a diverse array of gram-type negative bacteria, including *Acinetobacter* sp. strain ADP1. They are composed of repeating pilin subunits, which are initially synthesized as immature pre-pilin polypeptides. Features of the prepilin subunit include (a) a short, positively charged leader sequence, (b) an unusual amino acid as the first residue of the mature pilin (N-methylphenylalanine), and (c) a highly conserved and hydrophobic amino-terminal domain which is assumed to stack in a helical array forming the core of the pilus structure (Hobbs and Mattick, 1993). Many pilin-like proteins have been found which possess these characteristics, and which function in processes such as protein secretion, DNA transfer by conjugation and transformation, and the morphogenesis of filamentous bacteriophages. It is suggested that these pilin-like proteins function in the processing and export, as well as organization into fimbrial-like structures, of proteins resembling type IV pili. It appears likely that these complexes are related systems with a common

evolutionary origin, but which have diverged to fulfill a wide range of functions involving cell surface interactions and organization (Hobbs and Mattick, 1993).

The fifth gene, *comB*, which is also significantly similar to the type IV pilins, is required for natural transformation of ADP1. It does not appear to be involved in pilus biogenesis or function (Herzberg et al., 2000). Analysis of a chromosomally encoded *comB::lacZ* transcriptional fusion demonstrated that *comB* is expressed maximally during prolonged exponential phase and the stationary phase of growth. It was concluded that induction of competence after subculture into fresh medium is not due to the induction of gene expression, but rather dilution into fresh medium may provide the necessary energy for DNA uptake (Herzberg et al., 2000). From these data, a model for the transformation apparatus of ADP1 has been proposed. ComP, ComE, ComF, and ComB are suggested to form a heteropolymeric shaft spanning the outer membrane, periplasm, and inner membrane by which (or through which) DNA enters the cell. ComC may act at the cell surface, since it is required for DNA binding and/or uptake, but is not homologous to the type IV pilins. When energy is supplied, this apparatus enables DNA uptake by the cell in the single-stranded form (Herzberg et al., 2000).

Natural Transformation and the Techniques Used in These Studies

The most useful feature of the ADP1 transformation systemin the laboratory is the high efficiency of transformation and homologous recombination into the chromosome of linear DNA fragments for making directed chromosomal mutations. With this feature, I was able to use Polymerase Chain Reaction (PCR) methods to amplify specific DNA fragments of ADP1 sequence encoding engineered mutations for the purpose of replacing

the homologous chromosomal segment with modified alleles. This enabled me to construct a large number of strains encoding *benA* promoter regulatory region mutations in both wild-type and *benA::lacZ* transcriptional fusion backgrounds to investigate regulation and test our models.

As detailed in Chapter 2, the *Acinetobacter* transformation system allowed me to determine whether a carboxy-terminally His-tagged BenM (BenM-His) would function *in vivo* comparably to the wild-type BenM. For this analysis I replaced the corresponding wild-type *benM* of ADP1 with a PCR-generated DNA fragment encoding *benM-his*. I was then able to determine that *benM-his* functions as well as the wild-type *benM*, on the chromosome in an otherwise wild-type strain, in allowing the growth of ADP1 on benzoate as a sole carbon and energy source

As detailed in Chapter 3, the *Acinetobacter* transformation system enabled me to construct a series of chromosomally encoded *benA* promoter mutation strains. Promoter mutations were constructed on plasmids, and then a specific DNA fragment of ADP1 sequence carrying the mutations was amplified by PCR and used to transform ADP1. The resultant strains were then used to determine *in vivo* whether the *benA* promoter mutations affected growth with benzoate as the sole carbon and energy source. This approach was further utilized to construct of a series of chromosomally encoded *benA::lacZ* transcriptional fusion strains with the mutant *benA* promoters. These strains allowed me to assay the *in vivo* levels of *benA* expression from the mutant promoters, as compared to levels in the wild-type *benA::lacZ* background, in response to benzoate, CCM, or both. Exploitation of the ADP1 natural transformation system greatly

facilitated *in vivo* analysis of the chromosomal *benA* promoter mutations and improved our understanding of BenM-mediated *benA* transcriptional regulation.

CHAPTER 2

BENM-MEDIATED SYNERGISTIC INDUCTION OF THE *BENABCDE* OPERON OF *ACINETOBACTER* SP. STRAIN ADP1 IN RESPONSE TO THE INDUCERS BENZOATE AND *CIS,CIS*-MUCONATE[1]

[1]Bundy, B. M., L. S. Collier, T. Hoover, and E. L. Neidle. 2001. To be submitted to Cell.

Abstract

BenM, a LysR-type transcriptional regulator of *Acinetobacter* sp. strain ADP1, controls expression of *benABCDE*, an operon involved in benzoate degradation via the β-ketoadipate pathway. *In vivo* studies suggested BenM responds to either benzoate or *cis,cis*-muconate as inducer, and both compounds together have a synergistic effect on transcription. To investigate whether the effects of these inducers result from direct interactions with BenM, we utilized a run-off *in vitro* transcription assay and DNase I footprinting. These experiments demonstrated that the synergistic effects of benzoate and *cis,cis*-muconate require BenM and no additional *Acinetobacter* proteins. *In vitro* transcription assays indicated basal transcription in the absence of BenM, and a 2-fold repression of transcription in the presence of BenM without inducer. The presence of either inducer alone with BenM yielded activation. *cis,cis*-Muconate elicited a response 2-fold greater than benzoate as the sole inducer. In concert, the two inducers had a greater effect on transcription than the sum of their individual contributions. Based on DNaseI protection patterns, a model is presented in which there are three sites in the *benA* operator-promoter region which BenM may bind. In this model, the presence or absence of inducer determines which two of the three possible binding sites BenM will occupy. If either inducer is present alone, the DNA-protein complex shifts from an inactive to an active conformation in which BenM binds a different set of sites. Both compounds together are more effective than either alone in mediating this shift from the inactive to the active conformation. Therefore, both compounds in concert appear to induce conformational changes in the BenM-DNA complex that effect increased transcriptional activation.

Introduction

LysR-type transcriptional regulatory proteins (LTTRs) control prokaryotic gene expression in response to a wide array of environmental compounds. They form one of the largest families of homologous transcriptional regulators and are important to diverse aspects of prokaryotic physiology (Schell, 1993). Genome analysis of *Pseudomonas aeruginosa* PAO1 suggests that this bacterium alone may encode more than 100 of these regulators (Stover et. al, 2000).

The BenM protein of the soil bacterium *Acinetobacter* sp. strain ADP1 is a LTTR of a subclass that controls aromatic compound degradation by bacteria of at least four genera. An unusual feature of BenM-mediated regulation was suggested by studies utilizing chromosomal *lacZ* transcriptional fusions to assess expression of *benA*. BenM activated transcription of *benA* in response to either of two inducers, benzoate or *cis,cis*-muconate (CCM), and the presence of both compounds yielded much higher levels of gene expression than either compound alone (Collier et al., 1998). The apparent ability of two distinct inducers to act in a synergistic fashion with a single LTTR was novel and intriguing. However, the *in vivo* studies could not rule out possible contributions of additional *Acinetobacter* proteins. In the current studies, we purified BenM to investigate inducer-mediated transcriptional activation *in vitro*.

The deduced BenM amino acid sequence is highly homologous to that of CatM, a regulator also from *Acinetobacter* sp. strain ADP1. BenM and CatM are 59% identical and 75% similar at the amino acid level. Both BenM and CatM can interact with CCM to activate transcription of genes needed for catechol degradation, the *catBCIJFD* operon and *catA* (Collier et al., 1998; Romero-Arroyo et al., 1995). BenM, but not CatM, can

also activate *catA* expression by interacting with benzoate. Moreover, BenM responds to benzoate and/or CCM to activate expression of the *benABCDE* operon, which encodes enzymes for the conversion of benzoate to catechol. Under normal conditions, CatM is unable to activate *ben* gene expression (Collier et al., 1998).

DNA-binding activities of the LTTRs ClcR and CatR of *Pseudomonas putida* have been well studied. CatR and ClcR are homologues of CatM and BenM that regulate catechol/phenol and 3-chlorocatechol degradation, respectively (Parsek et al., 1995; McFall et al., 1998; Tover et al., 2000). CatR and ClcR are Class I transcriptional activators, contacting the C-terminal portion of the α-subunit of RNA polymerase (RNAP) (McFall et al., 1998). CatR regulates expression of the *catBCA* and *pheBA* operons in response to CCM, and ClcR regulates expression of the *clcABD* operon in response to 2-chloro-*cis,cis*-muconate (2-chloroCCM). CatR and ClcR bind to two distinct sites within their target promoter regions, the recognition and activation binding sites. The recognition site is occupied whether inducer is present or not, but the activation site is occupied by CatR only in the presence of inducer and by ClcR both in the presence and absence of inducer. Binding to the activation site allows productive contacts between the LTTR and RNAP to occur, leading to transcriptional activation (McFall et al., 1998; Tover et al., 2000).

The activation of *ben* gene transcription by BenM displays some similarities to the CatR/ClcR model of LTTR-mediated activation, but is complicated by the response to multiple inducers and the presence of a third possible LTTR-binding site. Here, we utilize *in vitro* transcription assays to demonstrate that BenM responds individually to both benzoate and CCM to activate transcription of *benA*. These assays demonstrate that

benzoate and CCM act synergistically to induce a level of *benA* transcription that is significantly higher than that achieved with equivalent amounts of either inducer alone. DNase I footprints of BenM at the *benA* promoter region confirm that the presence of both benzoate and CCM dramatically alters the pattern of protection from nuclease digestion. We present a model of BenM-mediated *benA* expression involving two possible configurations between BenM and the *benA* promoter region. Formation of these configurations is determined by inducer binding to BenM, and results in either the repression or induction of *benA* expression.

Results and Discussion

BenM is a Tetrameric LTTR

To facilitate purification of BenM, a hexahistidine tag was introduced at the carboxy-terminus of the protein (chimeric protein referred to as BenM-His). Before purifying and characterizing BenM-His, we determined if the hexahistidine tag affected BenM function *in vivo*. *Acinetobacter* sp. strain ADP1 is naturally transformable (Juni and Janik, 1969), and a DNA fragment encoding the chimeric protein was used to replace the corresponding chromosomal region in the *benM*-deleted strain ACN389. Successful allelic replacement was confirmed by sequence analysis and Southern hybridization, and the resulting *Acinetobacter* strain was designated ACN205. ACN205 utilized benzoate as the sole carbon source, growing with a generation time of approximately 90 min, which was comparable to the growth rate of the wild-type strain. Since strains lacking BenM are unable to grow on benzoate as the sole carbon source, these data showed that BenM-His activated *ben* gene expression *in vivo*.

BenM-His was expressed in *E. coli* and purified to homogeneity by affinity chromatography using a nickel chelating column. The size of the monomeric BenM-His from the deduced amino acid sequence, 45 kDa, was verified by denaturing polyacrylamide gel electrophoresis (data not shown). Analytical gel filtration was used to determine the native size of the protein. BenM-His eluted as a single peak with an apparent molecular weight of 180 kDa from the gel filtration column (data not shown). Inclusion of both benzoate and CCM at 0.5 mM in the gel filtration chromatography buffer had no effect on the mobility of BenM-His. Taken together, these data suggested that BenM-His exists normally as a tetramer and that the multimeric state of BenM-His remains unchanged upon binding inducer. This is consistent with data for other LTTRs, which are usually either dimeric or tetrameric (as discussed in Hryniewicz and Kredich, 1995).

BenM Regulates Transcription of *benA* and *benM In Vitro*

BenM-His protein was used in a single round *in vitro* transcription assay to investigate its response to inducer. The assay was developed with σ^{70}-saturated RNAP from *E. coli* and a linear DNA template containing a constitutive *benA* promoter mutation (Collier et. al., 1998) and the divergently transcribed *benM* promoter (Fig. 2.1). Transcripts were generated from the constitutive *benA* promoter in the absence or presence of BenM-His, but transcription from this promoter increased 2- to 3-fold when BenM-His and inducers were included in the assay (Fig. 2.2A). For quantitation of the *in vitro* transcription reactions, the amount of *benA* transcript was normalized to levels of the RNA1 transcript from the vector, which remained unchanged in each reaction.

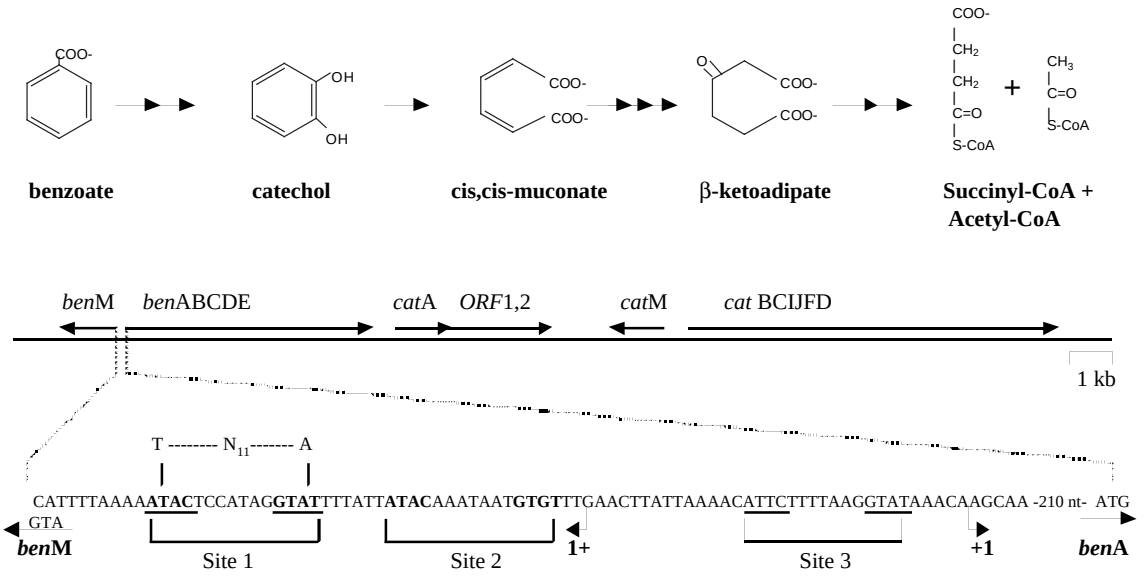


Figure 2.1. The β-ketoadipate Pathway of *Acinetobacter* sp. Strain ADP1 and the BenM-Regulated *benA* Promoter Regulatory Region. The organization of the *ben* and *cat* genes of *Acinetobacter* sp. strain ADP1 is shown with key compounds of the β-ketoadipate pathway displayed above. The sequence of the *benA* promoter region is displayed below. Transcriptional start sites for *benM* and *benA* are designated +1. The putative LTTR binding site with perfect dyad symmetry centered at –64, in relation to the *benA* transcriptional start site, designated Site1, is shown in bold and underlined. LTTR binding sites with one perfect half-site and one imperfect half-site, centered at -43 and –12 in relation to the *benA* transcriptional start site, Sites 2 and 3, respectively, are also indicated. Site 2 is shown in bold. Site 3 is underscored.

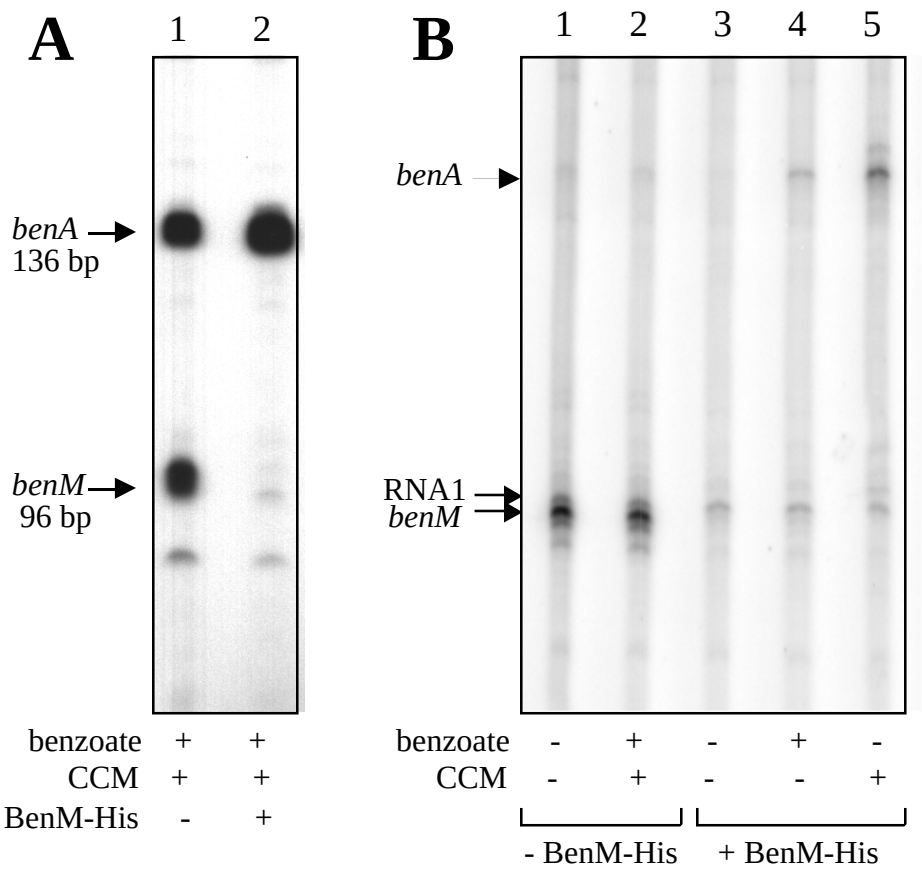


Figure 2.2. *In Vitro* Transcription Analysis of BenM-Mediated *benA* Expression.

In vitro transcription assays carried out using linear DNA templates that contained either a constitutive *benA* mutant promoter or the wild-type *benA* promoter. The *benA*, *benM* and RNA1 transcripts are indicated. (A) Transcripts from the constitutive *benA* promoter with and without BenM-His (lanes 1 and 2, respectively) in the presence of both benzoate and CCM (2 mM each). (B) Transcripts from the wild-type *benA* promoter without BenM-His (lanes 1 and 2) and with BenM-His (lanes 3-5). Inducers included (at 2 mM) in each reaction are indicated below each lane.

In addition to the *benA* promoter region, the DNA template contained the promoter for the divergently transcribed *benM*. A transcript of the size predicted for one originating from the *benM* promoter was observed when BenM-His was absent from the reaction. Consistent with the observation of negative autoregulation by most LTTRs (Schell, 1993), levels of transcript from the *benM* promoter were reduced 4-6 fold when BenM-His was present in the transcription reaction (Fig.2.2B).

Once assay conditions with the constitutive *benA* promoter were established, we examined the influence of BenM-His on transcription from the wild-type *benA* promoter *in vitro*. In the absence of BenM-His, transcription from the *benA* promoter was poor. When BenM-His was included in the transcription assay, without any inducer, the low level of transcription from the *benA* promoter was repressed approximately 2-fold (Fig. 2.2, lane 3). These data are consistent with previous *in vivo* β-galactosidase assays using a chromosomally encoded *benA::lacZ* transcriptional fusion (Collier et al., 1998).

Synergistic Induction of *benA* by BenM in Response to Benzoate and CCM

The effect of benzoate or CCM in the transcription assay was investigated with the wild-type promoter. As shown in Figure 2.3, the presence of 2 mM benzoate or 2 mM CCM increased BenM-His-mediated transcription from the *benA* promoter by approximately 1.5-fold and 3.3-fold, respectively. These data are consistent with the observation that CCM is more effective than benzoate in increasing *benA* expression *in vivo* (Collier et al., 1998).

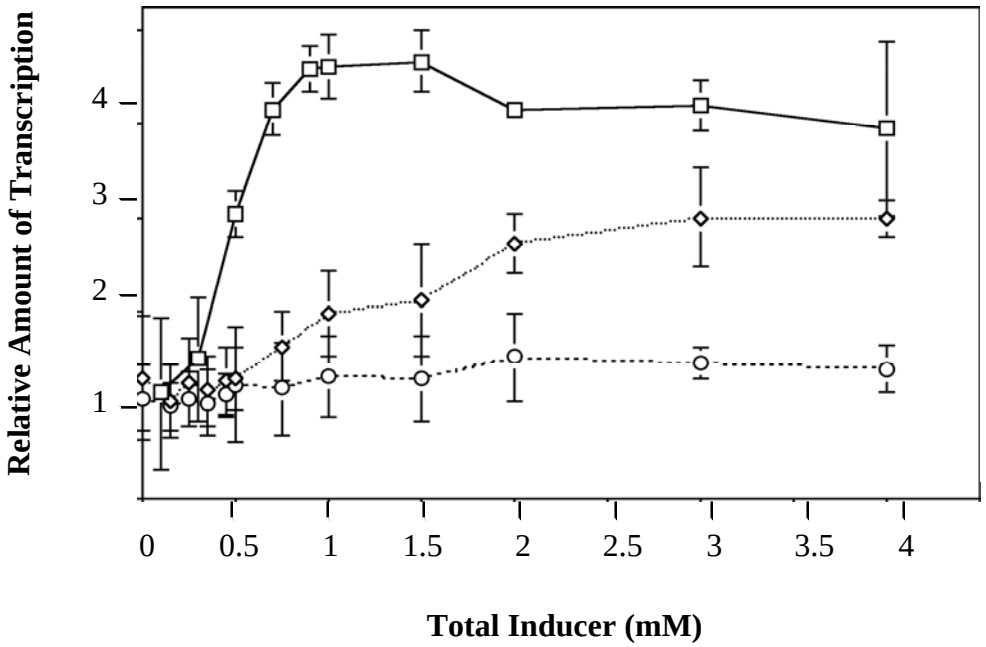


Figure 2.3. *In Vitro* Transcription Assays Showing Synergistic Induction of *benA* by BenM in Response to Benzoate and CCM. *In vitro* transcription products were analyzed with a Phosphorimager (Molecular Dynamics) using ImageQuant 1.2 to quantitate the data. The *benA* transcripts were normalized to the corresponding level of RNA1 transcript in each lane. Data shown are the result of three separate experiments and standard deviations are indicated. Circles and diamonds indicate the level of transcription initiation induced by benzoate or CCM alone, respectively. Boxes indicate transcription initiation mediated by BenM-His in the presence of equimolar amounts of benzoate and CCM. The total amount of inducer in each reaction is indicated on the X-axis (for example, at 0.5 mM total inducer there was 0.25 mM each of benzoate and CCM in the reaction containing both inducers).

The effect of benzoate and CCM together in the transcription assay was then examined. As shown in Figure 2.3, benzoate and CCM acted synergistically to stimulate BenM-mediated transcription from the *benA* promoter region. For example, at a concentration of 0.5 mM neither benzoate nor CCM significantly stimulated transcription, but when 0.25 mM each of benzoate and CCM (a total concentration of 0.5 mM inducer) were included in the transcription assay, *benA* expression increased approximately 3-fold. Although the synergistic effect of benzoate and CCM was most pronounced at low inducer concentrations, this effect was observed over the entire range of inducer concentrations that we examined.

Additional *in vitro* transcription reactions were performed in which one inducer was held at a constant concentration and the other titrated from 0 to 4 mM. Whether benzoate or CCM was held constant, the peak level of transcription occurred where both inducers were at or near equimolar concentrations (data not shown). Similar results were observed when native BenM rather than BenM-His was used in the *in vitro* transcription assay (data not shown).

Identification of Transcriptional Start Sites for *benA* and *benM*

To begin to address how BenM regulates transcription of the *ben* genes, we identified the *benA* and *benM* transcriptional start sites by primer extension methods. A labeled oligonucleotide primer complementary to the sequence that spanned 130 to 149 bp upstream of the *benA* translational start site was hybridized to RNA isolated from the wild-type strain grown under inducing or non-inducing conditions for *ben* gene expression. The oligonucleotide primed synthesis of a single 87 base cDNA extension

product with RNA from cells induced by the presence of benzoate and/or CCM, but not from cells grown in the absence of inducers (data not shown). The apparent transcriptional start site was an A residue located 216 nucleotides upstream of the *benA* initiation codon (Fig. 2.1). The same start site was observed regardless of whether benzoate, CCM or both compounds were present. This A residue coincided exactly with the previously predicted transcriptional start site, which was based on sequence comparison with the promoter of the CatM-regulated *catB* gene which is 56% identical over a 73 bp sequence to the *benA* promoter region (Collier et al., 1998). RNAse protection assays confirmed the presence of a single transcriptional start site for the *benABCDE* operon and verified the approximate location of this site (data not shown). The size of the *benA in vitro* transcription product also correlates with this transcriptional start site for *benA*.

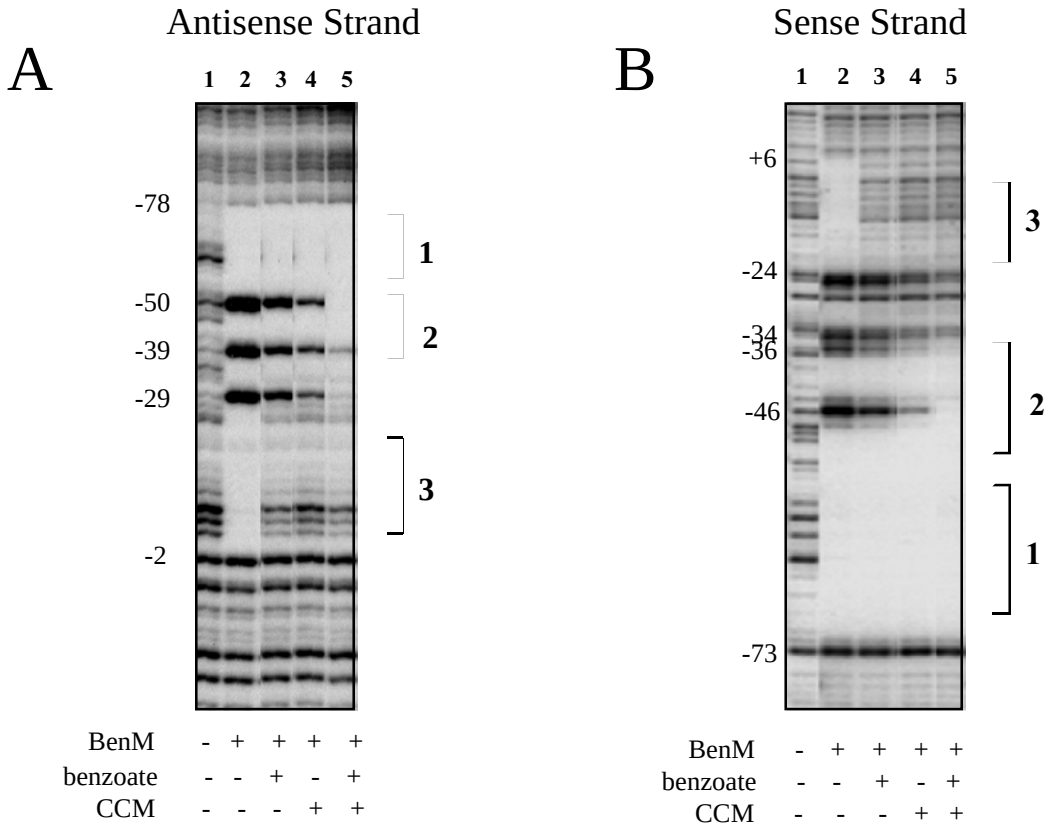
Conventional primer extension methods using total RNA isolated from induced cultures of ADP1 failed to detect transcription of *benM*. Presumably, negative autoregulation by BenM results in low levels of *benM* expression in wild-type cells. Therefore, we used the products from a non-radioactive *in vitro* transcription reaction carried out in the absence of BenM for a primer extension reaction. An oligonucleotide primer was used that was complementary to a sequence that covered 46 to 27 bases downstream of the *benM* translational start site. The oligonucleotide primed synthesis of a single 92 base cDNA extension product, suggesting that an A residue 46 bases upstream of the *benM* initiation codon is the transcriptional start site of *benM* (Fig. 2.1).

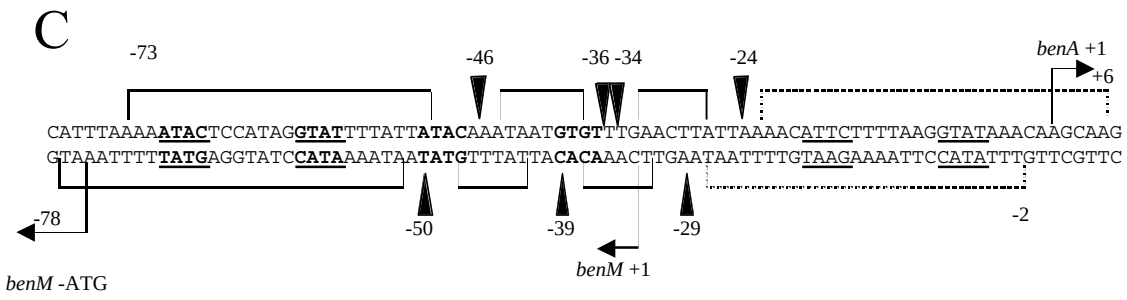
Interactions of BenM with the *benA* Promoter Region in the Absence of Inducer

To investigate interactions between BenM and the *benA* promoter region *in vitro*, native BenM was used for DNase I footprinting experiments. In the absence of inducers, BenM protected nucleotides –78 to –2, with respect to the *benA* transcriptional start site, on the antisense strand, and nucleotides –73 to +6 on the sense strand (Fig. 2.4, lanes 2A and 2B). Sites that were hypersensitive to DNase I cleavage were observed at –50, -39, and –29 on the antisense strand, and at -46, -36, -34, and –24 on the sense strand.

LTTRs belonging to the CCM-binding subclass, which includes BenM, recognize sequences with dyad symmetry similar to ATAC-N_7-GTAT (Schell, 1993; Coco et al., 1993). The region protected by BenM that was most distal to *benA* contained the sequence ATAC-N_7-GTAT centered at –64 (Fig. 2.4C). This apparent binding site for BenM was designated Site 1. The ATAC and GTAT half sites are 11 bp apart (center-to-center) and so they lie on the same face of the DNA helix. Additional sequences that resemble the ATAC-N_7-GTAT motif are found within the region of DNA that is protected by BenM. One of these is centered at –43 and has the sequence ATAC-N_7-GTgT (lower case letter indicates deviation from consensus sequence). This sequence, designated Site 2, may be another binding site for BenM. A third sequence, designated Site 3, with sequence ATtC-N_7-GTAT (lower case letter indicates deviation from consensus sequence) is centered at –12 and overlaps the *benA* promoter. Binding of BenM to Site 3 should repress transcription of *benA*, which is consistent with data from the *in vitro* transcription assays that showed BenM repressed basal *benA* expression in the absence of inducer.

Figure 2.4. DNase I Footprinting of BenM at *benA*. Protection from DNase I cleavage by BenM was assessed on the antisense (A) and sense (B) strands of the *benA* promoter region. Site 1, 2, and 3 are indicated with brackets to the right of A and B. For lanes 1, no BenM or inducers were included in the footprinting reactions. For lanes 2, BenM was included in the reactions, but no inducers were present. For lanes 3 and 4, reactions contained BenM and either 1 mM benzoate (lanes 3) or 1 mM CCM (lanes 4). Reactions that contained BenM in the presence of 0.5 mM each of benzoate and CCM (for a total of 1 mM inducer) are shown in lanes 5. (C) Graphical representation of the interactions of BenM at *benA*. Site 1 is indicated by bold and underscored type. Site 2 is indicated by bold type, and Site 3 is indicated by underscored type. Nucleotides protected from DNase I digestion in both the absence and presence of inducer compounds are indicated by solid brackets. Nucleotides protected only in the absence of inducer are indicated by dotted brackets. Solid triangles indicate sites that were strongly hypersensitive to DNase I digestion. These data were first described by Dr. L. Collier (Collier, 2000). I have obtained similar results.


A
Antisense Strand
1 2 3 4 5
-78
-50
-39
-29
-2
1
2
3
BenM - + + + +
benzoate - - + - +
CCM - - - + +
B
Sense Strand
1 2 3 4 5
+6
-24
-34
-36
-46
-73
3
2
1
BenM - + + + +
benzoate - - + - +
CCM - - - + +


C
-73
-46
-36 -34
-24
benA +1
+6
CATTTAAAAATACTCCATAGGTATTTTATTATACAAATAATGTGTTTGAACTTATTAAAACATTCTTTTAAGGTATAAACAAGCAAG
GTAAATTTTTATGAGGTATCCATAAAATAATATGTTTATTACACAAACTTGAATAATTTTGTAAGAAAATTCCATATTTGTTCGTTC
-78
-50
-39
-29
-2
benM +1
benM -ATG

As indicated earlier, the results from the gel filtration chromatography analysis of BenM-His suggested that BenM exists as a tetramer. Assuming BenM binds DNA as other transcriptional activators with helix-turn-helix (H-T-H) motifs (Pabo and Sauer, 1992; Hryniewicz and Kredich, 1995), we postulate that in the absence of inducer a BenM tetramer binds simultaneously to Sites 1 and 3 with a dimer binding to each ATAC-N_7-GTAT-like motif. These sites are 52 bp apart (center-to-center), which places them 5 turns of the DNA helix apart assuming 10.5 bp per turn. This means that Sites 1 and 3 are on the same face of the DNA helix, and the simultaneous binding of a BenM tetramer to these sites would likely result in looping of the intervening DNA. This DNA loop could account for the sites located between Sites 1 and 3 that are hypersensitive to DNase I cleavage.

BenM-DNA Interactions at *benA* are Altered in the Presence of Inducer

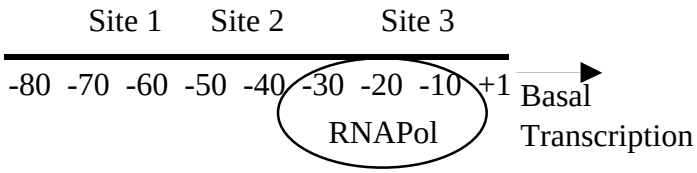
To determine whether inducers of *ben* gene expression have an effect on the DNase I protection pattern of BenM at *benA*, benzoate and CCM were added to the footprinting reactions. BenM-DNA complexes formed in the presence of benzoate or CCM had shorter footprints than in their absence (Fig. 2.4, Lanes 3 and 4). The region from -2 to -29 on the antisense strand and +6 to –24 on the sense strand were not fully protected from DNaseI cleavage in the presence of benzoate or CCM, indicating that BenM has reduced affinity for Site 3 in the presence of these inducers. This reduced affinity for Site 3 by BenM in the presence of inducers could enable RNAP access to the promoter, thereby relieving the repression caused by BenM in the absence of inducer. BenM continued to protect upstream regions near -80 in the presence of either inducer.

Hypersensitive sites were observed in the presence of either inducer at –50, -39, and –29 on the antisense strand, and –46, -36, and –24 on the sense strand (Fig. 2.4, Lanes 3 and 4). However, the degree of hypersensitivity was diminished relative to no inducer being present (Fig. 2.4, Lanes 1). The protection pattern of BenM at *benA* was essentially the same whether benzoate or CCM was present, except Site 3 appeared to be protected to a lesser degree and the hypersensitive sites were less reactive when CCM was present than with benzoate.

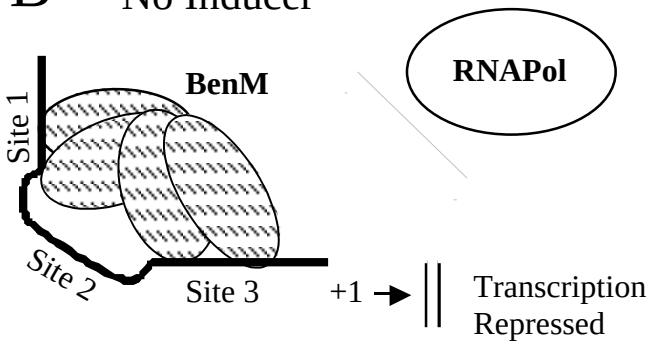
Given that benzoate and CCM act together to induce higher levels of *ben* gene expression than either compound alone, footprinting studies were conducted in which both inducers were present. When benzoate and CCM were both present in the binding reaction, the length of the footprint remained the same as that with either inducer alone, but cleavage of the hypersensitive site at position -39 was reduced even further than with either inducer alone (Fig. 2.4A and B, lane 5). In fact, the -50 and -46 hypersensitive sites, both contained within Site 2, were fully protected from DNaseI cleavage in the presence of both inducers. Thus, Site 2 appears to be protected only when both inducer compounds are present. A model for transcriptional regulation based on these results is shown in Fig. 2.5. These footprinting studies demonstrate that when present together, these inducers result in significant conformational changes in the BenM-DNA complex. The presence of both benzoate and CCM appears to determine which set of LTTR-binding sites BenM associates with in the *benA* promoter region. In the absence of inducer, BenM appears to assume a conformation that binds to sites 1 and 3. In this conformation, BenM represses transcription from *benA*.

Figure 2.5. Model of BenM-Mediated Activation of *benA*. (A) In the absence of BenM, RNA-Polymerase (RNAPol) initiates basal *benA* transcription. (B) BenM in the absence of inducer is in a conformation that prefers binding Sites 1 and 3, looping-out the intervening DNA and blocking RNAP from the promoter. (C) BenM in the presence of both benzoate and CCM assumes a conformation that binds Sites 1 and 2. Binding of Sites 1 and 2 by BenM allows RNAPol access to the *benA* promoter and may enable contacts between BenM and RNAPol for high-level *benA* transcription. The presence of both benzoate and CCM shifts the equilibrium of BenM from a conformation that binds Sites 1 and 3 to a conformation that prefers binding Sites 1 and 2. Benzoate or CCM alone only slightly shift the conformational equilibrium of BenM towards the conformation binding Sites 1 and 2. This slight shift towards the activating-conformation of BenM in the presence of either single inducer may not be enough to activate *ben* gene expression to a level able to support growth on benzoate, but does relieve the repression of *benA* caused by BenM in the absence of inducer.

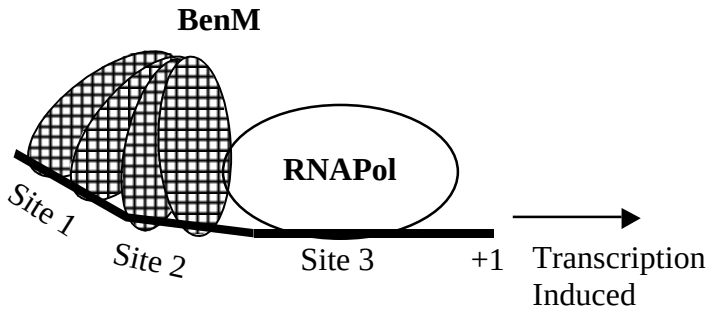
A No Activator



B No Inducer



C Activator and Both Inducers



In the presence of either benzoate or CCM, a second conformation is stabilized that favors binding to Sites 1 and 2, which results in transcriptional activation from the *benA* promoter. Under such conditions, however, some BenM appears to remain in the repressor conformation, and so full transcriptional activation is not achieved. We do not know if this is because the inducer-binding sites in BenM are not saturated with inducer or if binding of a single inducer to BenM is insufficient to lock the protein into the activation conformation. The presence of both benzoate and CCM appears to shift the equilibrium towards the activation conformation further than either inducer alone, resulting in full transcriptional activation from the *benA* promoter.

This is reminiscent of the 'light-switch' model for AraC-mediated activation of the genes necessary for arabinose utilization in *E. coli* (Schleif, 2000; Harmer et al., 2001). AraC has an amino-terminal arm of 18 residues that extends from the inducer-binding domain and binds the DNA-binding domain in the absence of the inducer arabinose. In the absence of inducer, AraC is thus locked into a conformation that favors binding to two well separated sites in the *ara* gene promoter region, I_1 and O_2, causing looping-out of the intervening DNA and repression of *ara* gene expression. When arabinose is bound to AraC, the amino-terminal arm shifts around more freely, partially contacting the DNA-binding domain and partially contacting the arabinose binding site. Free movement of the arm allows AraC to adopt a conformation that favors binding the two closely spaced sites, I_1 and I_2, just upstream of the *ara* genes and inducing transcription. Since AraC is already bound to the promoter region in the absence of inducer, response to the presence of inducer can occur extremely rapidly, constituting a shift from binding sites I_1 and O_2 to I_1 and I_2 (Schleif, 2000; Harmer et al., 2001).

Binding of BenM to Site 2 would position it upstream of and partially overlapping the –35 region of the *benA* promoter, a placement characteristic of Class I transcriptional activators which contact the carboxy-terminus of the α-subunit of RNAP (Ishihama, 1993). Other LTTRs that function as Class I transcriptional activators have been described, and they include OxyR (Tao et al., 1993), TrpI (Gussin et al., 1992), CysB (Shi and Bennett, 1994), CatR (Chugani et al., 1997), and ClcR (McFall et al., 1997).

Interactions Between CatM and the *benA* Promoter Region

CatM, another regulator of CCM degradation in *Acinetobacter* sp. strain ADP1, is 59% identical and 75% similar to BenM at the amino acid level. BenM and CatM both regulate expression of the *catA* and *catBCIJFD* operons in response to CCM, and BenM can regulate *catA* expression in response to benzoate. Despite their homology and overlapping regulatory roles, CatM cannot activate *ben* gene expression suitable for growth of strain ADP1 on benzoate (Collier et al., 1998). Previous *in vivo* studies suggested that CatM binds the *benA* promoter region, since the basal level of *benA* transcription is increased approximately 4-fold in a strain that lacks both BenM and CatM, as compared to the wild-type strain (Cosper et al., 2001).

To test the ability of CatM to regulate *benA* expression, a recombinant form of CatM with a hexahistidine at the carboxy-terminus (CatM-His) was used in *in vitro* transcription assays. CatM-His repressed *benA* transcription in the absence of inducer to a level similar to that observed with BenM (Fig. 2.6, Bar 2). CatM-His did not respond to the presence of benzoate in the transcription assay (up to 3 mM), consistent with previous *in vivo* data (Fig. 2.6, Bar 3) (Collier et al., 1998).

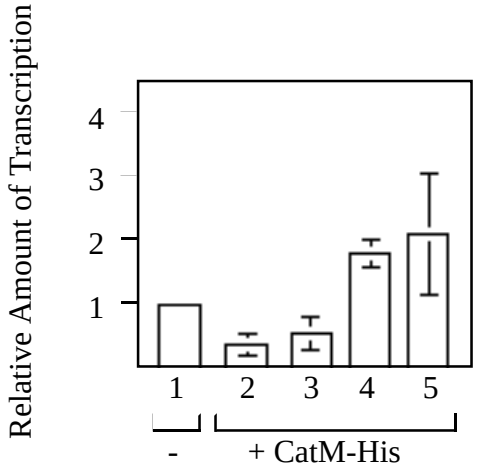


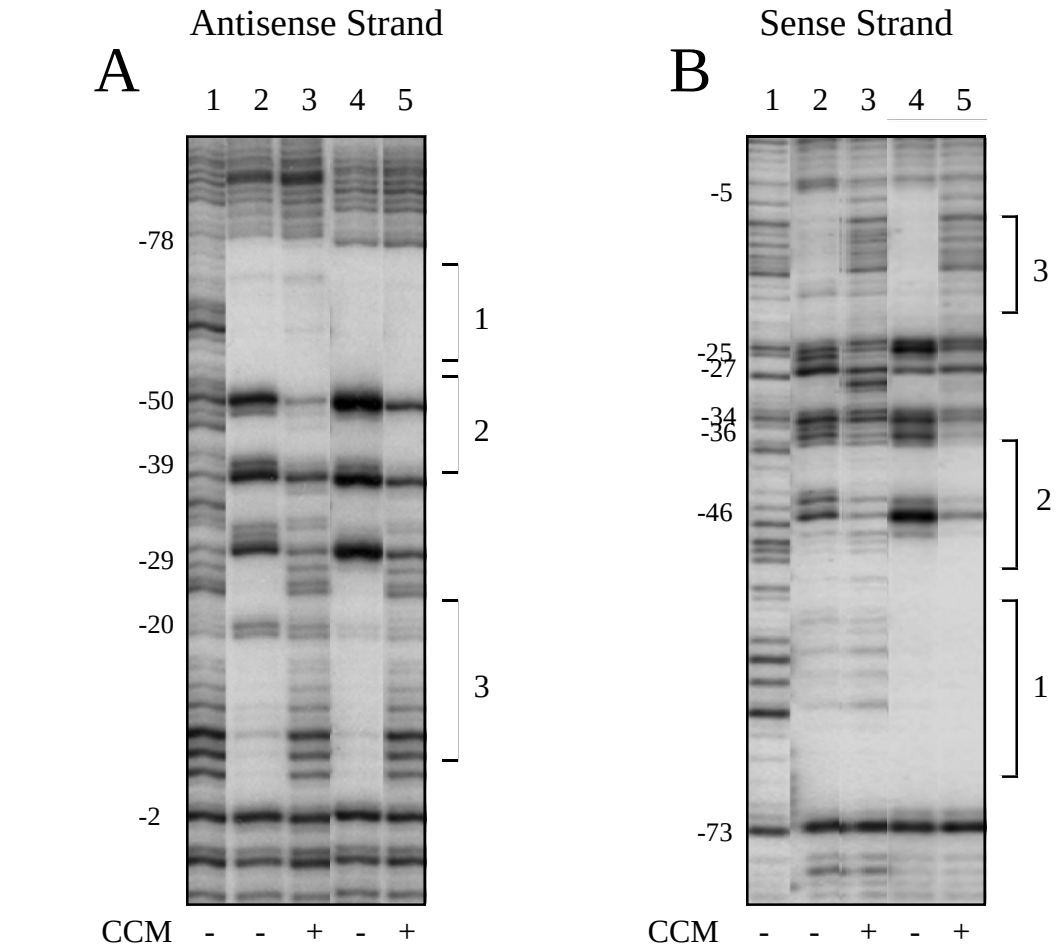
Figure 2.6. *In Vitro* Transcription Analysis of *benA* Activation Mediated by CatM. *In vitro* transcription assays were performed with the wild-type *benA* promoter fragment and CatM-His. Transcripts were analyzed with a phosphorimager (Molecular Dynamics) using ImageQuant 1.2 to quantitate the data. Transcripts were normalized to the corresponding RNA1 in each lane. Bar 1: basal *benA* transcription in the absence of activator. Bars 2: Transcriptional repression of *benA* in the presence of CatM-His in the absence of inducer. Bars 3, 4, and 5: *benA* transcription mediated by CatM-His in the presence of either 1 mM benzoate (Bar 3), 1 mM CCM (Bar 4), or 0.5 mM each inducer (Bar 5).

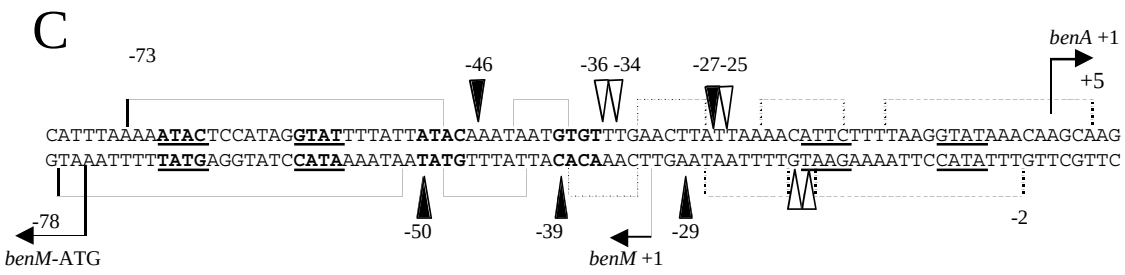
In the presence of 1 mM CCM (Fig. 2.6, Bar 4) CatM-His activated transcription slightly from the *benA* promoter region (~1.8 fold). CatM-His did not appear to demonstrate a significant synergistic response to the presence of both benzoate and CCM (0.5 mM each) in the transcription assay (Fig. 2.6, Bar 5). The level of *benA* transcription induced by CatM in response to CCM was comparable to that induced by BenM in response to benzoate.

The binding of native CatM to the *benA* promoter region was examined in DNase I footprinting assays. In the absence of inducer, CatM protected a region from -78 to -2 and -73 to +5, on the antisense and sense strands, respectively (Fig. 2.7). As suggested for BenM, the protection of the *benA* promoter –10 region by CatM in the absence of inducer suggests that it also represses transcription from *benA* by blocking access of RNAP to the *benA* promoter. Similar to the footprint with BenM, hypersensitive sites were observed in footprints with CatM at -50, -39, and -29 on the antisense strand and at -46, -36, -34, and –27, -25 on the sense strand. Additional hypersensitive sites at –19 and –20 on the antisense strand, however, were observed in the footprint with CatM.

Since CatM binds the *benA* promoter region in a manner similar to BenM in the absence of inducer, we investigated the effect of inducer on CatM binding to the *benA* promoter region. Including up to 5 mM benzoate in the footprinting assay had no effect on binding of CatM to the *benA* promoter region (data not shown). In the presence of CCM, however, the protection pattern of CatM at *benA* was almost indistinguishable from that of BenM. The addition of both benzoate and CCM to the footprinting assay did not significantly alter the footprint of CatM at *benA*, as compared to that in the presence of CCM alone.

Figure 2.7. DNase I Footprinting of CatM at *benA*. Protection from DNase I cleavage by CatM was assessed on the antisense (A) and sense (B) strands of the *benA* promoter region. Sites 1, 2, and 3 are indicated with brackets to the right of A and B. For lanes 1, no CatM or inducers were included in the footprinting reactions. For lanes 2, CatM was included in the reactions, but no inducers were present. For lanes 3, reactions contained CatM in the presence of 1 mM CCM (lanes 4). For lanes 4 and 5, the corresponding reactions with BenM, without inducer (lane 4) or with 1 mM CCM (lane 5), are shown for comparison. (C) Graphical representation of the interactions of CatM at *benA*. Site 1 is indicated by bold and underscored type. Site 2 is indicated by bold type, and Site 3 is indicated by underscored type. Nucleotides protected from DNase I digestion in both the absence and presence of inducer compounds are indicated by solid boxes. Nucleotides protected only in the absence of inducer are indicated by dotted boxes. Solid triangles indicate sites that were strongly hypersensitive to DNase I digestion. Open triangles indicate sites that were less hypersensitive to DNase I cleavage. These data were first described by Dr. L. Collier (Collier, 2000). I have obtained similar results.


Antisense Strand
A
1 2 3 4 5
-78
-50
-39
-29
-20
-2
1
2
3
CCM - - + - +
Sense Strand
B
1 2 3 4 5
-5
-25
-27
-34
-36
-46
-73
3
2
1
CCM - - + - +


C
-73
-46
-36 -34
-27-25
benA +1
+5
CATTTAAAAATACTCCATAGGTATTTTATTATACAAATAATGTGTTTGAACTTATTAAAACATTCTTTTAAGGTATAAACAAGCAAG
GTAAATTTTTATGAGGTATCCATAAAATAATATGTTTATTACACAAACTTGAATAATTTTGTAAGAAAATTCCATATTTGTTCGTTC
-78
-50
-39
-29
-2
benM-ATG
benM +1

Though BenM and CatM are 59% identical and both activate transcription of the *cat* genes, the inability of CatM to respond to benzoate explains why CatM cannot activate transcription of the *ben* genes to levels able to support growth on benzoate under normal conditions. The level of *benA* expression induced by CatM in response to CCM was most similar to that of BenM in response to benzoate (data not shown). Since the level of CatM-mediated expression of *benA in vivo* in response to CCM is too low to support growth on benzoate, we postulate that BenM-mediated expression of *benA* in response to benzoate alone would similarly be too low to support growth on benzoate. This suggests that the synergistic effect of benzoate and CCM on BenM-mediated expression of *benA* is required for growth of strain ADP1 on benzoate. The basal level of *ben* gene expression observed in the *in vitro* transcription assay would allow some CCM to be made when benzoate is available, which would enable BenM to turn on *ben* gene expression to a high level and thus allow growth on benzoate.

Additional Discussion

In addition to BenM, other transcriptional activators in the CCM-binding LTTR subclass may respond to more than one inducer. For example, CbnR is a LTTR that regulates the degradation of 3-chlorobenzoate in *Ralstonia eutropha* NH9 in response to both CCM and 2-chloroCCM. Data also suggest that CbnR, like BenM, may respond to benzoates (Ogawa et al., 1999). Similarly, CbeR, a LTTR that regulates 3- and 4-chlorobenzoate degradation in *Burkholderia* sp. NK8 appears to respond to benzoate, as well as 3- and 4-chlorobenzoate (Francisco et al., 2001).

There are other LTTRs, not of the CCM-binding subclass, which also seem to respond to multiple inducer compounds. These include IlvY which appears to respond to α-acetolactate, α-acetohydroxybutyrate, and α-acetohydroxyacid (Wek and Hatfield, 1988). AlsR appears to respond to changes in pH and also acetate (Ramos et al., 2000). The LTTR XapR normally responds to xanthosine, deoxyinosine, and deoxyadenosine, to different extents, as inducers. Mutational studies of XapR selecting for response to different purine nucleosides have shown that single residue changes in the putative inducer binding domain, as determined by alignment with the CysB crystal structure, can cause XapR to either have a very broad substrate recognition or alter its specificity for certain purine nucleosides (Jorgensen and Dandanell, 1999).

In addition, some transcriptional regulators that are not LTTRs respond to multiple inducer compounds. These include *P. putida* BkdR, which responds to L-valine, L-leucine, L-isoleucine and D-leucine (Madhusudhan et al., 1999), and *E. coli* GalR which responds to D-galactose and D-fucose (Adhya, 1989). Another interesting example is the tet-repressor. Tet-repressor responds to tetracycline-chelated Mg^{2+} as inducer. Under physiological pH, tetracycline has a high affinity for divalent metal ions. It was demonstrated that induction requires both the teracycline, which serves a role in specificity of binding, and the Mg^{2+} ion, which serves as the actual inducer (Orth et al., 1999). Of all these examples of possible multiple inducer compound, none report experiments using combinations of the possible inducer compounds. As observed with BenM, some of these regulators that recognize multiple inducers may respond synergistically to combinations of effector molecules, but this has yet to be demonstrated.

Protein-protein synergism in transcriptional regulation has been demonstrated in a number of prokaryotic and eukaryotic systems (Ptashne and Gann, 1997). This type of synergy can occur by a single protein making multiple contacts with a second protein, often RNAP, or by two separate activator proteins contacting a third protein, again usually RNAP (Ptashne and Gann, 1997). In contrast to the protein-protein synergy, BenM responds to the simultaneous presence of two distinct inducer compounds to induce a level of transcription far greater than with either inducer alone. A potential advantage to this inducer-inducer activation may be that it enables an extremely sensitive fine-tuning mechanism for the regulation of gene expression.

Experimental Procedures

Bacterial Strains, DNA Isolation, and Growth Conditions

The *Acinetobacter* strain ADP1, also known as BD413 (Juni and Janik, 1969), was used for total RNA isolations. ADP1 was grown overnight in 5 ml minimal medium with 3 mM benzoate, then diluted into 60 ml fresh medium. This culture was grown to an OD_{600} of about 0.6, fresh benzoate added to 4 mM, and the cultures grown an additional 45 min before harvesting and isolating RNA using Tri Reagent (Sigma) as above. *Escherichia coli* DH5α (Gibco BRL) was used as a plasmid host. *E. coli* were cultured in Luria-Bertani (LB) broth, while *Acinetobacter* strains were grown in a minimal medium as previously described (Shanley et al., 1986). Unless indicated otherwise, 3 mM benzoate served as the sole carbon source for *Acinetobacter* strains. Antibiotics were added as needed at the following concentrations: tetracycline (Tc), 6

ug/ml; kanamycin (Km), 25 ug/ml; ampicillin (Ap), 150 ug/ml; zeomycin (Zeo), 50 ug/ml. Standard methods were used for plasmid purifications, restriction enzyme digestions, electrophoresis, ligations, and *E. coli* transformations (Sambrook et al., 1989).

Strain Construction

The *benM*-deleted strain ACN389 was constructed by allelic replacement of the wildtype *benM* with linear DNA containing a 359 bp *benM* deletion from 1930 to 2289. Growth was selected on benzoate in the presence of 0.5 mM Succinate, and the resultant colonies were checked by southern hybridization.

The chromosomally encoded *benM-His* strain ACN205 was constructed by allelic replacement with a PCR product encoding the *benM*-6xHis tag. The PCR fragment was generated by first performing two separate but overlapping PCR reactions for DNA upstream or downstream of the 6x-his insertion. Primers incorporated the sequence for the 6x-his tag so that the upstream product encoded the 6x-his at its 3' end, and the downstream product encoded the 6x-his at its 5' end (Primer set #1; 5'GCTAGTATT-AATGACGGGAAT3' and 5'TCAGTGGTGGTGGTGGTGGTGCCAGTTTGGCGG-CTCAGT3': Primer set #2; 5'GGAACAGCGTGCGTTTAGTAC3' and 5'CACCACCACCACCACCACTGACAAAAAACCCATTAGACT3'.). The overlapping 6x-his sequences would then hybridize in a third PCR (containing 2 ul of each initial PCR) to give a combination template with the 6x-his located in the middle. The outermost primers were used to amplify the full-length hybridized template and the resulting DNA fragment was used to replace the corresponding region of the *benM*-

deleted ACN389. Growth on benzoate was used as the selection, and the strain was then confirmed by Southern hybridization and sequence analysis.

Primer Extension Analysis of *benA* and *benM*

A primer extension assay was used to determine the transcriptional start site of *benA*. *Acinetobacter* sp. strain ADP1 was grown in 5ml LB in the presence of 3 mM benzoate. After overnight growth, cells were harvested by centrifugation and total RNA was extracted using TRI reagent (Sigma) according to the manufacturers protocol. An oligonucleotide primer, 5'-CGAATATCCATTTCAGCTTT-3' (Genosys Biotechnologies), that was complementary to sequences 130-149 bases upstream of the putative *benA* translational start site was end labeled with [γ-^{32}P]ATP (Amersham Pharmacia Biotech) using T4 polynucleotide kinase (Promega). Unincorporated [^{32}P]ATP was removed by passage through a MicroSpin G-25 column (Amersham Pharmacia Biotech). Extension reactions with the labeled primer and purified RNA were carried out using the Promega Primer Extension kit according to the manufacturers instructions. The cDNA products were analyzed on a Stratagene Castaway^{TM} 8% polyacrylamide, 7M urea precast sequencing gel. To localize the transcriptional start site, sequencing reactions were performed simultaneously with the *fmol* DNA Cycle Sequencing kit (Promega) using the same labeled oligonucleotide primer as above and a plasmid containing the wild-type *ben* region as template.

Primer extension was also used to determine the transcriptional start site of *benM* using RNA from a non-isotopic *in vitro* transcription reaction. An oligonucleotide primer, 5'-GCTCCTCAACCACAGCCACA-3' (Genosys Biotechnologies),

complementary to sequences 43 to 24 bases upstream of the *benM* translational start site, was end-labeled as above and extension reactions were performed with RNA from the non –isotopic transcription reaction. Sequencing reactions using this labeled primer were performed as for *benA* to localize the transcriptional start site.

RNAse Protection of *benA*

For RNAse protection assays, a 423 base RNA probe extending from 345 bases upstream, to 27 bases downstream, of the *benA* translational start codon was generated using the MAXIscript kit according to the manufacturers instructions (Ambion). The probe was biotin-labeled using the BrightStar Psoralen-Biotin kit according to the manufacturers instructions (Ambion). 350 ng of labeled probe was hybridized with 25 μg of RNA, treated with RNAse, and products visualized using the RPAII kit (Ambion) according to the manufacturers instructions.

Purification of 6x-His-Tagged BenM and CatM

To construct carboxy-terminally 6x-his-tagged BenM and CatM, the corresponding genes were amplified by PCR using primers that incorporated a 5' *Nde*I site and a 3' *Xho*I site. Primers for *benM* were 5'TCAATTCATATGGAACTTA-GACATCTCCGC3' and 5'TCAATTCTCGAGCCAGTTTGGCGGCTCAGTAAA3'. Primers for *catM* were 5'TCAATTCATATGGAACTAAGACACCTCAGA3' and 5'TCAATTCTCGAGTTCGATGAGTGGCCTGATATG3'. PCR products were cloned into pET21b (Promega) and designated pBAC433 and pBAC430, respectively. *E. coli* strain BL21DE3 cells expressing *benM* from pBAC433, or *catM* from pBAC430, were

grown overnight in 60 ml LB with 150 μg/ml Ap and diluted the following day into 4 liters of fresh medium. Cultures were grown to an OD600 of 0.2 and induced with 0.2 mM IPTG during overnight growth. The next day cells were harvested by centrifugation, sonicated in 15-20 second bursts for a total of 3 minutes, and the extract clarified by centrifugation as above. Clarified cell extract was applied to a Hi-Trap 5 ml metal chelating column (Pharmacia Biotech) charged with nickel and proteins were eluted as per the manufacturers instructions.

Partial Purification of BenM and CatM

For partial purification of BenM and CatM, the corresponding genes were amplified by PCR with primers that incorporated a 5' *Bgl*II site, and a 3' *Eco*RI or *Hin*dIII site, respectively. Primers for *benM* were 5'TCAATTAGATCTATGCAT-TGCACAGCCGCCCTT3' and 5'ATAGAATTCTTAGCTGGCTTTGGGTTTAAG-TAAAAT3'. Primers for *catM* were 5'TCAATTAGATCTATGGAACTAAGAC-ACCTCAGA3' and 5'AGCGGATAACAATTTCACACAGGA3'. The PCR products with *benM* and *catM* were cloned into the *lac* based expression vector pLAC11 (Warren et al., 2000) and designated pBAC191 and pBAC67, respectively. *E. coli* strain ALS225 (Warren et al., 2000) cells expressing *benM* from pBAC191, or *catM* from pBAC67, were grown overnight in 150 ml LB with 150 ug/ml Ap and diluted the following day into 6 liters fresh medium. Cultures were grown 4 h at 37°C with shaking before 1 mM IPTG was added. Cultures were grown an additional 4 h, harvested by centrifugation, and the cell pellet stored at -70°C for later use. Cell pellets were suspended in 30 ml buffer A (50 mM Tris, pH 8.0, 5 mM dithiothreotol (DTT), 10% glycerol), and a crude

cell extract prepared by sonication as described above.. The crude extract was clarified by centrifugation at 10,000 x g for 45 min. Ammonium sulfate was added to 35% saturation to the resulting supernatant, and the precipitated proteins collected by centrifugation at 12,000 x g for 30 min. The pellet was suspended in 7 ml buffer A and loaded onto a 5 ml HiTrap Desalting column (Amersham Pharmacia Biotech) then onto a 5 ml HiTrap Heparin column (Amersham Pharmacia Biotech). The column was washed with 50 ml buffer A at a rate of 1 ml/min and bound protein was eluted with a 60 ml 0 to 1 M NaCl gradient in buffer A. BenM eluted at approximately 400 mM NaCl. CatM eluted at approximately 600 mM NaCl.

DNase I Footprinting

For footprinting experiments, the *benAM* intergenic region from 411 to 130 bases upstream of the *benA* translational start site was amplified by PCR and cloned into pUC19, generating pBAC366. For labeling of the antisense strand, plasmid pBAC366 was digested with *Eco*RI, dephosphorylated with calf intestinal alkaline phosphatase (Promega), and the 5' ends phosphorylated with [γ-^{32}P]ATP (Amersham) by T4 polynucleotide kinase for 30 min at 37°C. The DNA fragment was then digested with *Hin*dIII to release a singly end-labeled 248 bp operator/promoter fragment and purified as described previously (Hoover et al., 1990). For labeling of the sense strand, a *Hin*dIII deletion of pBAC366 was generated and designated pBAC373. pBAC373 was digested with *Hin*dIII, end labeled with [γ-^{32}P]ATP, and a 221 bp operator/promoter fragment purified following digestion with *Eco*RI as described above. The labeled sense and

antisense fragments were incubated overnight with various concentrations of BenM with or without benzoate and/or CCM, and DNase I footprinting reactions carried out as previously described (Wang and Hoover, 1997).

In Vitro Transcription Assays

In vitro transcription assays (17.5 µl, final volume) were performed essentially as described (Chugani et al., 1997) with exceptions as noted. For DNA template, the *benAM* intergenic region from 411 to 148 bases upstream of the *benA* translational start site was amplified by PCR, cloned into pMP7 (Hershberger et al., 1995), and designated pBAC346. pBAC400 is identical to pBAC346, except that it contains a single point mutation at position –8 with respect to the *benA* transcriptional start site. This G to A transition causes constitutive *benA* expression (Collier et al., 1998). 10nM PBAC346, or pBAC400, singly digested with *Hin*dIII was used as DNA template. Reactions contained template, 20 U RNAse inhibitor (Promega), 0.2 mg of acylated bovine serum albumin (Promega) per ml, 1ug poly[dI-dC], 0.4U *E. coli* σ-70 RNA polymerase holoenzyme (Epicentre Technologies) in a buffer consisting of 2.7 mM DTT, 0.4 mM EDTA, 0.75 mM Tris, 50 mM NaCl, 10 mM MgCl2, 6.7 mM sodium phosphate, and 10% glycerol in 40 mM HEPES (pH 7.5), and BenM (3 µM) alone or with benzoate and/or CCM added at the concentrations indicated. Transcript formation was quantitated using a Phosphorimager (Molecular Dynamics) and ImageQuant 1.2. *benA* transcripts were normalized to the RNA1 transcript present in all reactions.

Analytical Gel Filtration Chromatography

Analytical gel filtration was performed with a Superose 12 HR 10/30 column (Amersham Pharmacia Biotech). The buffer contained 20 mM Tris, 1 mM EDTA, 1 mM DTT, 150 mM NaCl, and 5% glycerol (pH 8.5). The column was calibrated using the Sigma molecular weight standards 12,000 to 200,000 kit as per the manufacturers specifications (Sigma). The calibrating proteins used were Cytochrome C (12,400 MW), Carbonic Anhydrase (29,000 MW), Bovine Albumin (66,000 MW), Alcohol Dehydrogenase (150,000 MW), and β-Amylase (200,000 MW). 0.8 mg BenM-His was loaded and the column washed at a rate of 0.37 ml per minute. Protein elution was followed by monitoring the absorbance at 280 nm.

CHAPTER 3

MUTATIONAL ANALYSIS OF THE *BENABCDE* PROMOTER OF *ACINETOBACTER* SP. STRAIN ADP1: TESTING THE MODEL OF BENM-MEDIATED *BENA* EXPRESSION

Introduction

The experiments described in Chapter 2 led to a proposed model for BenM-mediated transcriptional activation of *benA*. According to this model, BenM binds to Sites 1 and 2 to activate transcription when both inducers are present (as shown in Fig. 2.5 C). In the absence of inducer, BenM binds to Sites 1 and 3 and represses *benA* expression (Fig. 2.5 B). In this chapter, studies are described that test various aspects of this model.

A *benA* promoter mutation (allele *benMA 5146*), conferring CCM-mediated *ben* gene expression in a *benM*-mutated strain, was previously reported (Collier et al., 1998). This mutation is a T to A transversion at -40, with respect to the *benA* transcriptional start site. This position is immediately 5' of the 3' half-site of BenM-binding Site 2. In accordance with our model, this T to A mutation, which makes Site 2 more similar to the corresponding region of the CatM-regulated *catB* promoter, might enable CatM to contact *benA* promoter Sites 1 and 2 in response to CCM in a manner similar to BenM in response to benzoate and CCM together. Here, the effect of this mutation on the ability of CatM to bind to the *benA* promoter was investigated by DNase I footprinting.

In previous studies, a constitutive *benA* promoter mutation was described and analyzed by β-galactosidase analysis of a chromosomal *benA::lacZ* transcriptional fusion strain (Collier et al., 1998). This mutation is a single G to A transition at -8, with respect to the *benA* transcriptional start site (allele *benMA 5147*). This base is located in the 3' half-site of BenM-binding Site 3 of the *ben* gene promoter regulatory region. It was possible that this point mutation weakened the LTTR-DNA interactions of BenM and CatM at Site 3. Thus, the mutation might cause partial relief of the 2-fold repression of basal *benA* transcription normally caused by the LTTRs binding to the promoter and limiting the access of RNAP to this regulatory region. As described in this chapter, DNase I footprinting assays were utilized to investigate this possibility.

In order to test predictions that the LTTR-binding Sites 1 and 2 of our model are required for BenM-activated *ben* gene expression, site-directed *benA* promoter mutations were constructed. DNA promoter fragments were constructed *in vitro* in which the sequence of both half-sites of the dyad symmetry of Sites 1 and 2 were altered. In these two constructs, 6 bp of the intervening DNA between the elements of dyad symmetry were altered to create an *Eco*RI restriction site for ease of mutantion identification. These mutations completely altered the sequence of the binding regions, removing all dyad symmetry. However, the number of bases in these regions were the same as in the wild-type, and spacing of the regions relative to the promoter was not changed. To investigate additional aspects of the sequence of Site 2 in BenM-mediated *ben* gene expression, two mutant promoters were constructed in which only the 5’ half of dyad symmetry was mutated, or the intervening region between the half-sites was mutated.

Again, these mutations were constructed such that they created an *Eco*RI restriction site for ease of mutation identification.

Normally, the center of Sites 1 and 2 are 21 nucleotides apart, or two turns of the DNA helix. The center of Sites 1 and 3 are 52 nucleotides apart, or 5 turns of the DNA helix. Therefore, BenM proteins bound to Sites 1, 2, and 3 would be predicted to be on the same face of the DNA helix. To determine the effects of altering the spacing of the binding sites, *benA* promoter LTTR-binding site spacing mutations were constructed. It is assumed that insertion of 5 bp between two LTTR-binding sites, or insertion of one-half a helical turn of the DNA assuming 10.5 bases per turn, would position the downstream binding site on the opposite face of the DNA helix. Insertion of 11 bp would add a full turn of the DNA helix, keeping the sites on the same face but placing the sites farther apart. If spacing of the binding sites is important, then both 5 and 11 bp insertions might hinder BenM binding to the *benA* promoter, as well as hinder BenM-mediated *ben* gene expression. On the other hand, if the face of the DNA helix that the binding sites are positioned on is most important for proper contacts between BenM and the *benA* promoter, then insertion of 11 bp would not be expected to alter significantly BenM-mediated *ben* gene expression. I therefore constructed mutant *benA* promoters with 5 and 11 bp insertions between Site 1 and 2, and between Sites 2 and 3.

In the studies presented in this chapter, I utilized DNase I footprinting analysis of the plasmid-borne mutant promoters described above to determine their effects on BenM-binding to the *benA* promoter. In order to determine if these mutations affected growth of ADP1 on benzoate as a sole carbon and energy source, I utilized the natural transformation system of ADP1 to replace the corresponding *benA* promoter region of a

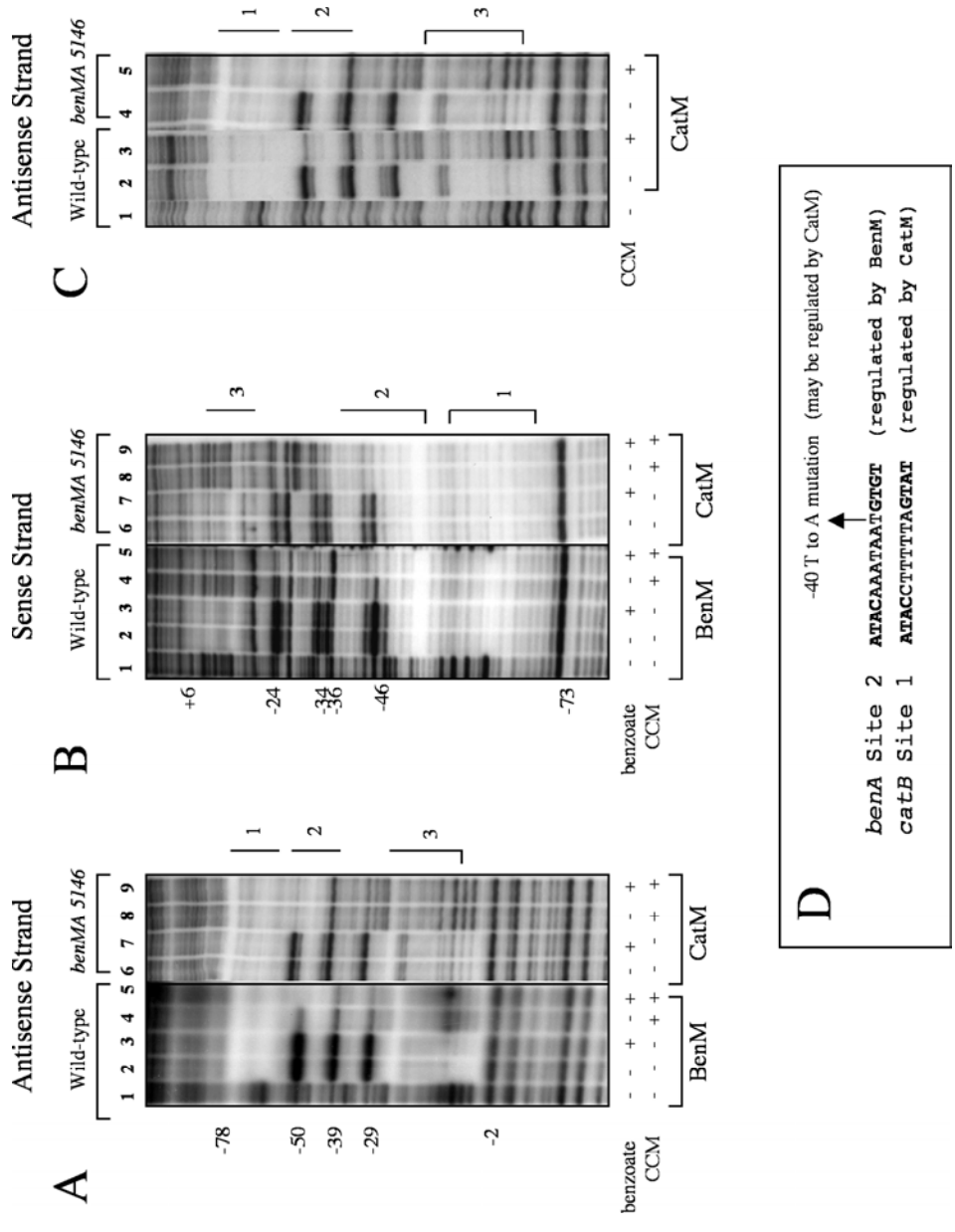
benM::sacB strain with the desired mutations. The resulting strains differ from the wild-type only in the sequence of the *benA* promoter region. Using this strategy, I also placed the promoter mutations in a strain with a chromosomal *benA::lacZ* transcriptional fusion. In the resulting strains, the promoter mutations controlled expression of the chromosomal *lacZ* marker, allowing β-galactosidase assays to analyze the effects of the promoter mutations *in vivo*.

Results and Discussion

Analysis of a *benA* Promoter Mutation Conferring CCM-Mediated *ben* Gene Expression in a *benM*-Mutated Strain

A previously described *benA* promoter mutation conferred CCM-inducible *ben* gene expression in a *benM*-mutated strain, *benMA 5146* (Collier et al., 1998). This mutation is a T to A transversion at position –40, with respect to the *benA* transcriptional start site, and is located in Site 2 of the *benA* promoter. Since the *benMA 5146* mutation makes Site 2 more similar to the Site 1 region of the CatM-regulated *catB* promoter, it might enable CatM to regulate *ben* gene expression in response to CCM alone. Since CatM is predicted to activate expression of *benMA 5146*, DNase I footprinting was used to compare the interactions of this regulator and the mutant promoter with those of BenM at the wild-type *benA* (Fig. 3.1, panels A and B). In the absence of CCM, each LTTR protected regions near Sites 1 and 3 from DNase I digestion and caused some sites to be hypersensitive to cleavage (Panels A and B, lanes 1-2 and lanes 6-7). These patterns of DNase I cleavage were similar for the mutant and wild-type promoters.

Figure 3.1. Comparison of the Ability of BenM and CatM to bind to the Wild-Type and *benMA 5146* -40 CCM-Inducible Promoters. DNase I footprinting analysis of the previously described -40 CCM-inducible *benMA 5146* mutant promoter on the (A) Antisense and (B) Sense strands. (A and B) Lane 1 contains the wild-type promoter DNA-fragment and no regulatory protein. Lanes 2 through 5 (wild-type promoter) contain 3 pmol BenM-His and either (Lane 2) no inducer, (Lane 3) 1 mM benzoate, (Lane 4) 1 mM CCM, or (Lane 5) 0.5 mM each benzoate and CCM. Lanes 6 through 9 (*benMA 5146* mutant promoter) contain 3 pmol CatM-His and either (Lane 6) no inducer, (Lane 7) 1 mM benzoate, (Lane 8) 1 mM CCM, or (Lane 9) 0.5 mM each benzoate and CCM. LTTR-binding Sites 1, 2, and 3 are indicated to the right with brackets. (C) Direct comparison of CatM-His binding at the wild-type and *benMA 5146* mutant promoters in the absence (Lanes 2 and 4) and presence (Lanes 3 and 5) of CCM on the antisense strand. Lane 1 contains no CatM-His. The approximate positions of LTTR-binding Sites 1, 2, and 3 are indicated to the right. (D) Comparison of the BenM-regulated *benA* Site 2 and the corresponding CatM-regulated *catB* Site 1. The –40 T to A transversion is indicated by an arrow.


A
Antisense Strand
Wild-type
benMA 5146
1 2 3 4 5 6 7 8 9
-78
-50
-39
-29
-2
1
2
3
benzoate
CCM
BenM
CatM
B
Sense Strand
+6
-24
-34
-36
-46
-73
C
Antisense Strand
CCM
D
-40 T to A mutation (may be regulated by CatM)
benA Site 2 ATACAAATAATGTGT (regulated by BenM)
catB Site 1 ATACCTTTTTAGTAT (regulated by CatM)

However, in contrast to BenM at the wild-type promoter, the addition of CCM caused CatM at the mutant promoter to confer the pattern of DNase I protection associated with full activation of *benA* (Compare Panels A and B, Lanes 4 and 8). This pattern of protection is provided by BenM only in the presence of both CCM and benzoate (Panels A and B, lane 5). The addition of benzoate had no effect on the DNase I footprint of CatM at the mutant promoter either in the presence of CCM (Panels A and B, Lanes 8 versus 9) or in the absence of CCM (Panels A and B, Lanes 6 versus 7).

A recent study analyzed spontaneous mutations in the *catB* structural gene that increased CCM-regulated *benA* expression in a strain lacking BenM. Elevated CCM levels, due to a less-active muconate cycloisomerase, appeared to increase the intracellular CCM to a level that enables CatM-mediated *ben* gene transcription in an otherwise wild-type strain (Cosper et al., 2000). These elevated CCM levels might enable *ben* gene expression by CatM in response to CCM at levels approximately equal to those mediated by BenM in response to both inducers. Since basal *ben* gene expression would not produce elevated CCM levels, it appears likely that the inability of CatM to respond to benzoate is why it is normally unable to activate *ben* gene expression in a *benM*-disrupted strain.

A direct comparison of CatM-His binding to the wild-type and -40 mutant promoters on the antisense strand is provided in figure 3.1C. As shown in Lanes 4 and 5 as compared to lanes 2 and 3, CatM better protects the site at –50 (Site 2) on the mutant promoter than on the wild-type promoter. BenM-His contacts with the wild-type and -40 mutant promoters were indistinguishable (data not shown). The *benMA 5146* mutation in Site 2 appears to enable CatM in response to CCM to contact Sites 1 and 2 in a manner

similar to BenM in response to both benzoate and CCM. These contacts between CatM and the *benMA 5146* promoter appear to allow CatM to activate *ben* gene expression to a level high enough to support growth on benzoate as a sole carbon and energy source.

Analysis of a Constitutive *benA* Promoter Mutation

A previously described constitutive *benA* promoter mutation*, benMA 5147*, contains a G to A transition at position –8, with respect to the *benA* transcriptional start site (Collier et al., 1998). This location is within LTTR binding Site 3, which causes repression of *ben* gene expression when bound by BenM or CatM. In order to test whether this constitutive mutation affects LTTR-binding of Site 3, DNase I footprinting analysis was performed on the wild-type and *benMA 5147* promoters. As shown in figure 3.2, various dilutions of BenM-His protected both the wild-type and *benMA 5147* promoters from DNase I digestion. BenM appears to bind strongly to Site 1 in both the wild-type and constitutive promoters, although at the lowest BenM concentrations there is some DNase I cleavage of the Site 1 region (Fig. 3.2 lanes 4-5 A and B). In the Site 3 region, BenM-His concentrations that protect the wild-type promoter from DNase I cleavage (Panel A, lanes 1 and 2) may allow greater DNase I cleavage of the mutant promoter (Panel B, lanes 1 and 2). Sites that may be more sensitive to DNase I cleavage on the constitutive promoter fragment than on the wild-type fragment are seen at -12, -19, and -20 (Fig. 3.2, lanes B2-B5). Nevertheless, BenM can clearly protect the Site 3 region of *benMA 5147* from DNase I digestion. The ability of BenM to bind simultaneously to the Site 1 and Site 3 regions of the mutant promoter is supported by the sites of

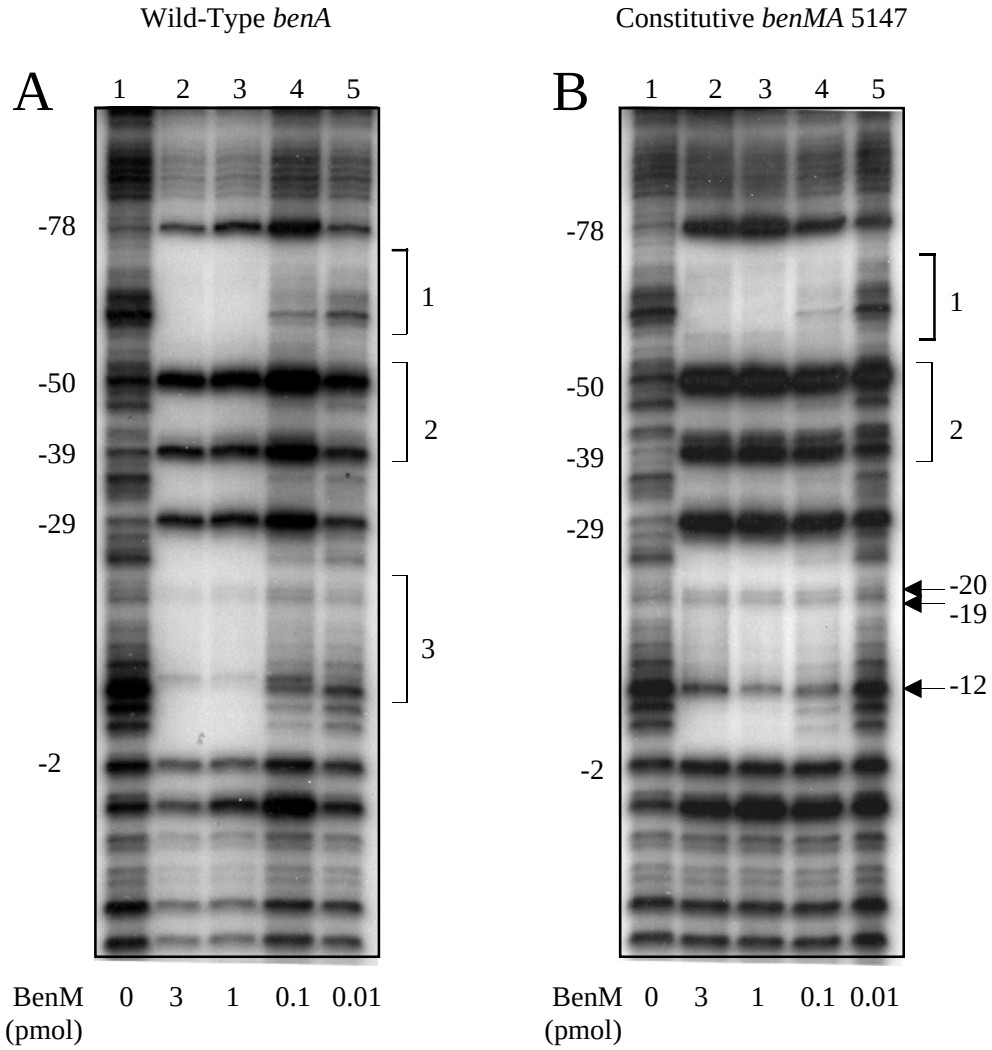


Figure 3.2. Comparison of the Ability of BenM to Bind to the Wild-Type *benA* and Constitutive *benMA 5147* Promoters. DNase I footprinting analysis of (A) wild-type and (B) -8 constitutive *benMA 5147* promoter fragments with (lane 1) no BenM-His, (lane 2) 3 pmol BenM-His, (lane 3) 1 pmol BenM-His, (lane 4) 0.1 pmol BenM-His, and (lane 5) 0.01 pmol BenM-His. The approximate positions of BenM-binding Sites 1, 2, and 3 are indicated to the right by brackets. Arrows indicate sites more sensitive to cleavage by DNase I on the constitutive promoter as compared to the wild-type promoter.

hypersensitivity to DNase I cleavage at positions –29, -39, and –50. DNase I footprinting of CatM at the constitutive promoter was similar to that of BenM (data not shown).

We had predicted that the constitutive promoter mutation would lessen the binding of BenM and CatM to Site 3 to help relieve the repression caused by the regulators in the absence of inducer. RNAP would then have better access to the *benMA5147* promoter than to the wild-type promoter. However, the conditions used in these footprinting experiments do not demonstrate conclusively that the mutation significantly weakens the affinity of BenM or CatM for Site 3. It may be that this mutation at –8 only slightly weakens BenM and CatM binding, but that its primary effect is to increase the affinity of RNAP for the promoter. This latter effect was clearly demonstrated in the *in vitro* transcription assays shown in Chapter 2.

BenM-Binding Sites 1 and 2 are Required for BenM-mediated *benA* Transcriptional Activation

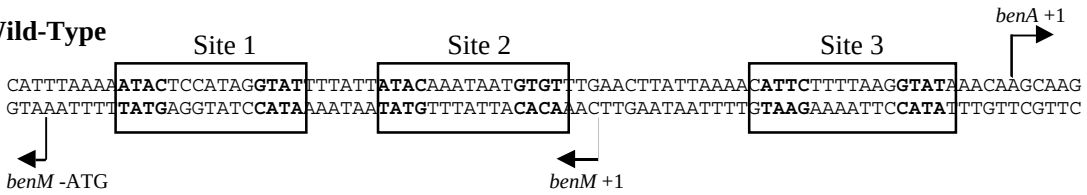
To test our model of BenM-mediated *ben* gene expression, *benA* promoter Site 1 and Site 2 mutations were constructed (See Table 3.1 for strain, plasmid, and allelic designations). These mutations are described in figure 3.3. I utilized the Altered Sites II *in vitro* Mutagenesis System (Promega) to construct a series of plasmids with the desired mutations. Our model of BenM-mediated *ben* gene expression as presented in Chapter 2 assumes that BenM binds to Site 1 in the presence or absence of inducer. Site 1 best agrees with the LTTR-binding site consensus sequence proposed for the CCM- and chloro-CCM-binding subclass of LTTRs (Schell, 1993; Coco et al., 1993). This site may be required for cooperative BenM-binding to Sites 2 and 3.

Table 3.1. Strains and Plasmids Carrying Site Directed *benMA* Promoter Mutations. The plasmid number and allelic designation of each site directed promoter mutation constructed for this study is listed. Corresponding ACN Strain numbers in both the wild-type and *benA::lacZ* transcriptional fusion backgrounds are given. The DNA fragments with the site-directed mutations are all in the pALTER II vector, supplied with the Altered Sites II *in vitro* Mutagenesis System (Promega), except for *benMA5467* and *benMA 5466* which are in pUC19. The sequences of the primers used for site directed mutagenesis are listed to the right. Plasmids used for DNase I footprinting on the antisense and sense strands are also given.

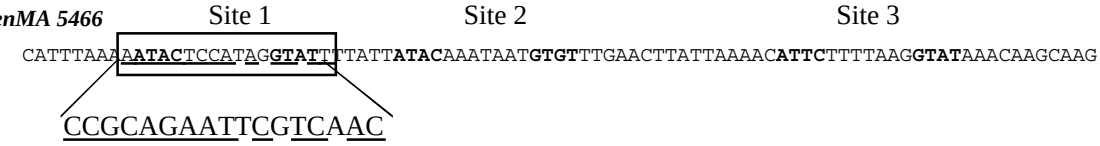
Allele	Designation	Plasmid	Strain	Strain with *benA::lacZ*	Footprint (Anti-Sense)	Footprint (Sense)	Primer Sequence 5'-3'
benMA 5466	Mutant 1G	pBAC522	ACN466	ACN461	pBAC513	pBAC518	TCTAAGTTCCATTTAAACCGCAGAATTCGTCAACTTATTATACAAA
benMA 5440	Mutant 2G	pBAC489	ACN440	ACN460	pBAC514	pBAC515	CTCCATAGGTATTTTATCCGCAGAATTCGTCAACTGAACTTATTAA
benMA 5438	Mutant 2B	pBAC486	ACN438	ACN435	pBAC490	pBAC517	TAGGTATTTTATGAATTCAATAATGTGTTT
benMA 5439	Mutant 2C	pBAC487	ACN439	ACN436	pBAC494	pBAC516	ATTTTATTATACGAATTCTGTGTTTGAACT
benMA 5437	Insert 5-12	pBAC483	ACN437	ACN434	pBAC491	pBAC485	ATACTCCATAGGTATTTTATTTTATTATACAAATAATGTGTT
benMA 5465	Insert 11-12	pBAC478	ACN465	ACN463	pBAC480	pBAC492	ATACTCCATAGGTATTTTATTGAATTCTTATTATACAAATAATGTGT
benMA 5467	Insert 5-23	pBAC521	ACN467	ACN464	pBAC481	pBAC519	AATAATGTGTTTGAACTTATTTTATTAAAACATTCTTTTAAG
benMA 5433	Insert 11-23	pBAC479	ACN433	ACN459	pBAC482	pBAC493	AATAATGTGTTTGAACTTATTGAATTCTTATTAAAACATTCTTTTAA

Figure 3.3. Sequences of the Site Directed Mutations Created in Sites 1 and 2. The DNA sequence of the wild-type *benA* promoter and the four site-directed *benA* promoter mutations created in Sites 1 and 2 are given. Sites 1, 2, and 3 are indicated by boxes on the wild-type sequence, and by bold type on the mutant promoter sequences. The divergently transcribed *benA* and *benM* transcriptional start sites, designated +1 on each strand, respectively, are indicated by arrows on the wild-type sequence. The *benM* ATG translational start codon is also indicated with an arrow on the wild-type sequence. The region affected by site-directed mutagenesis is indicated by a solid box for each mutant, and the sequence of the mutation created is displayed below. Mutated bases are indicated by underscoring.

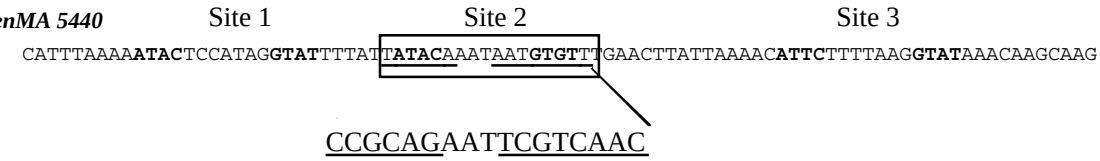
Wild-Type



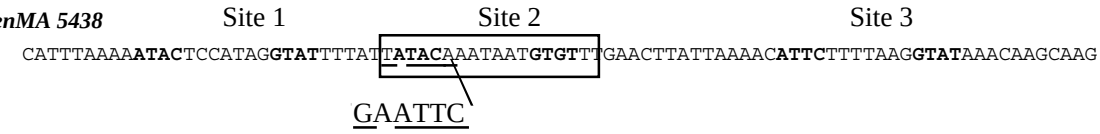
Mutant 1G
benMA 5466



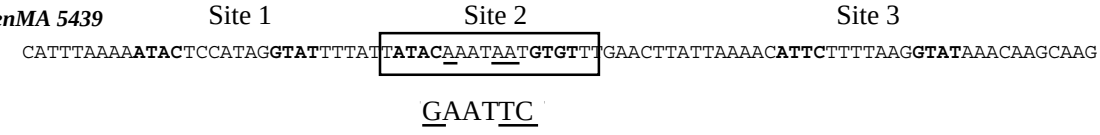
Mutant 2G
benMA 5440



Mutant 2B
benMA 5438



Mutant 2C
benMA 5439



To determine if Site 1 were required for BenM binding to the *benA* promoter, the sequence of Site 1 was specifically altered. This Site 1 mutation, referred to as Mutant 1G or *benMA 5466*, eliminates the elements of dyad symmetry and also places an *Eco*RI restriction site in the intervening DNA for ease of mutation identification. The spacing of Site 1 was preserved, and only the sequence of the bases was altered in *benMA 5466*. DNase I footprinting analysis on the *benMA 5466* promoter was performed. As shown in Figure 3.4E and Figure 3.5E, the mutation of Site 1 significantly hindered binding of BenM to the *benA* promoter. The *benMA 5466* fragments were not strongly protected by BenM from DNase I cleavage on either the sense (Fig. 3.4E) or antisense strands (Fig. 3.5E). We would expect the failure of BenM binding to a *benMA 5466* strain to cause low-level constitutive *benA* expression since, as discussed in Chapter 2, binding of BenM and CatM in the absence of inducer causes repression of *benA* expression.

I constructed a chromosomally-encoded *benMA 5466 benA::lacZ* transcriptional fusion strain, designated ACN461 to test *benA* expression *in vivo*. β-galactosidase activity of ACN461 was compared to that in strain ACN32, in which the wild-type *benA* promoter controls expression of *lacZ*. The mutation of Site 1 caused approximately 4-6 fold derepression of *benA* expression in the absence of inducers (data not shown). There was no activation of *benA* expression in ACN461. This agrees with previous *in vivo* data suggesting that basal *ben* gene expression increases approximately 4-fold in a *catM* and *benM* deleted strain (Cosper et al., 2000). In the strain lacking CatM and BenM there is no LTTR-binding to the *benA* promoter, and thus no repression of *ben* gene expression. Likewise, since there is no LTTR-binding of the *benA* promoter in the *benMA 5466* strain, there is no repression of *ben* gene expression.

Figure 3.4. Analysis of the Ability of BenM to Bind to the Site 1 and 2 *benMA* Promoter Mutations on the Antisense Strand. DNase I Footprinting analysis of the (A) wild-type, (B-D) Site 2 mutations, and (E) Site 1 mutation described in Fig. 3.3. Lanes 1 contain no BenM-His. Lanes 2 contain 3 pmol BenM-His with no inducer. Lanes 3 contain 3 pmol BenM-His with 1 mM benzoate. Lanes 4 contain 3 pmol BenM-His with 1 mM CCM. Lanes 5 contain 3 pmol BenM-His and 0.5 mM each benzoate and CCM. The approximate location of BenM-binding Sites 1, 2, and 3 are indicated to the right. The corresponding *benMA* allele and the sequence of the mutant promoter are shown below.

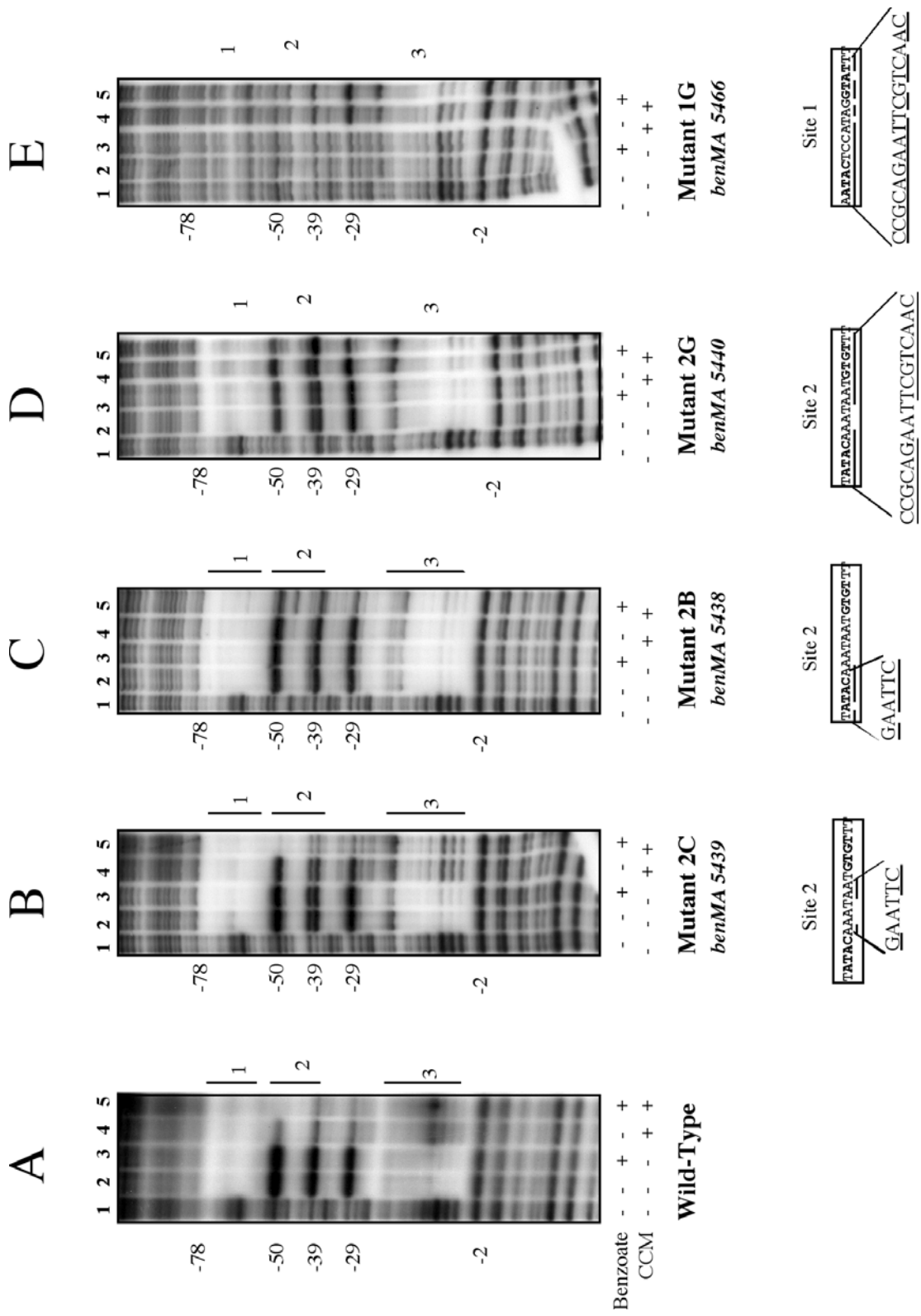
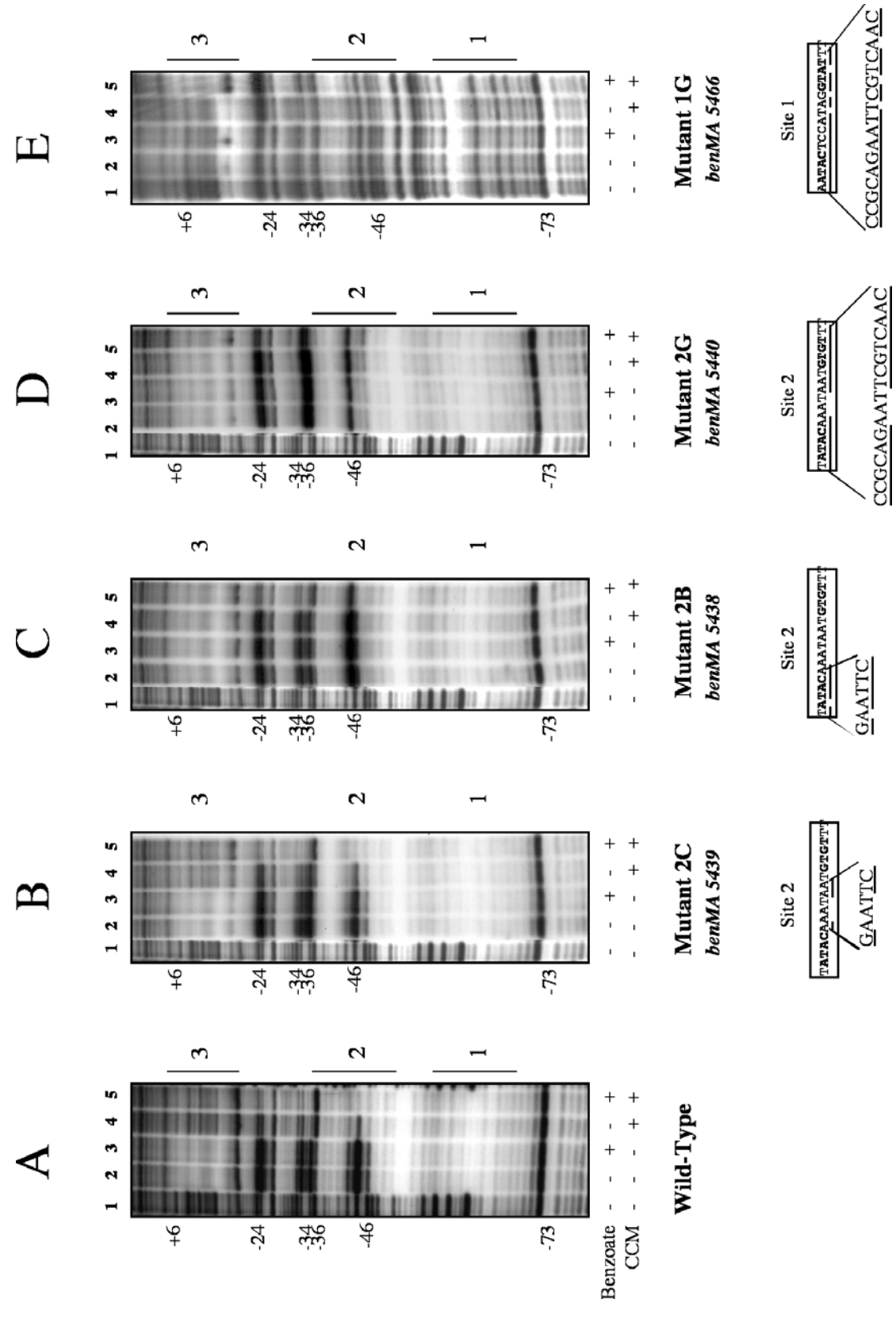

A
B
C
D
E
1 2 3 4 5
-78
-50
-39
-29
-2
1
2
3
Benzoate - - + - +
CCM - - - + +
Wild-Type
Mutant 2C
benMA 5439
Mutant 2B
benMA 5438
Mutant 2G
benMA 5440
Mutant 1G
benMA 5466
Site 2
TATACAAATAATGTGTT
GAATTC
Site 2
TATACAAATAATGTGTT
GAATTC
Site 2
TATACAAATAATGTGTT
CCGCAGAATTCGTCAAC
Site 1
AATACTCCATAGGTATT
CCGCAGAATTCGTCAAC

Figure 3.5. Analysis of the Ability of BenM to Bind to the Site 1 and 2 *benMA* Promoter Mutations on the Sense Strand. DNase I Footprinting analysis of the (A) wild-type, (B-D) Site 2 mutations, and (E) Site 1 mutation described in Fig. 3.3. Lanes 1 contain no BenM-His. Lanes 2 contain 3 pmol BenM-His with no inducer. Lanes 3 contain 3 pmol BenM-His with 1 mM benzoate. Lanes 4 contain 3 pmol BenM-His with 1 mM CCM. Lanes 5 contain 3 pmol BenM-His and 0.5 mM each benzoate and CCM. The approximate location of BenM-binding Sites 1, 2, and 3 are indicated to the right. The corresponding *benMA* allele and the sequence of the mutant promoter are shown below.


A
B
C
D
E
1 2 3 4 5
+6
-24
-34
-36
-46
-73
3
2
1
Benzoate - - + - +
CCM - - - + +
Wild-Type
Mutant 2C
benMA 5439
Mutant 2B
benMA 5438
Mutant 2G
benMA 5440
Mutant 1G
benMA 5466
Site 2
TATACAAATAATGTGTT
GAATTC
Site 2
TATACAAATAATGTGTT
GAATTC
Site 2
TATACAAATAATGTGTT
CCGCAGAATTCGTCAAC
Site 1
AATACTCCATAGGTATT
CCGCAGAATTCGTCAAC

Therefore, Site 1 appears to be required for BenM or CatM to bind to the *benA* promoter regulatory region. Consistent with the inability of BenM to activate *ben* gene expression, an otherwise wild-type strain with the *benMA 5466* allele in the chromosome, Strain ACN466, was unable to grow on benzoate as a sole carbon source.

Site 2 mutations were also constructed. Plasmid, strain, and allelic designations are shown in Table 3.1. Site 2 mutation sequences are detailed in figure 3.3. The Site 2 sequence was completely altered in *benMA 5440*, also referred to as mutant 2G, in which an *Eco*RI restriction site was inserted. I then constructed a mutation where only the 5' half-site of Site 2 dyad symmetry was altered, referred to as Mutant 2B or *benMA 5438*. This half-site mutation changes 6 bp to encode an *Eco*RI site. A similar mutation was constructed where 6 bp between the Site 2 half-sites of dyad symmetry were altered to encode an *Eco*RI restriction site. This mutation is referred to as Mutant 2C, or *benMA 5439*.

DNase I footprinting was performed on these three Site 2 mutated promoters. DNase I footprinting of the *benMA 5439* promoter is shown in figure 3.4B and 3.5B. The alteration of this region to encode an *Eco*RI restriction site resulted in only 3 altered bases. These three bases are not part of the ATAC-N_7-GTAT dyad symmetry. However, a single mutation in this vicinity (ATAC-N6-A-GTAT) significantly altered the interactions of CatM at the *benA* promoter (See Fig. 3.1). Therefore, these bases between the elements of dyad symmetry may be important for BenM-DNA contacts and BenM-mediated *benA* expression. As shown in Figure 3.4B and 3.5B, the *benMA 5439* promoter only slightly affected BenM-binding in response to no inducer (lane B2), either

inducer (lanes B3 and B4), or both benzoate and CCM (lane B5) on the antisense strand and sense strands as compared to BenM on the wild-type promoter (Fig. 3.4A and 3.5A).

In order to determine the effects of this Site 2 mutation on *benA* expression *in vivo*, I constructed a strain, designated ACN436, with the *benMA 5439* allele controlling expression of a chromosomal *benA::lacZ* fusion. As shown in figure 3.6, *benA* expression in ACN436 is lowered as compared to strain ACN32 which has a wild-type *benA* promoter. The pattern of *ben* gene expression in ACN436 in response to inducer is conserved. Therefore, the bases between the Site 2 half-sites may be important for full-level BenM-mediated *benA* expression. An otherwise wild-type strain with the *benMA 5439* allele, ACN439, grew on benzoate as the sole carbon source comparable to the wild-type strain ADP1.

DNase I footprinting of BenM at the *benMA 5438* promoter is shown in Figure 3.4C and 3.5C, on the antisense and sense strands, respectively. Mutation of the 5' half-site resulted in the loss of protection of Site 2 in response to both benzoate and CCM (Fig. 3.4 and 3.5, lanes C5 as compared to lanes A5). Evidence for this stems from the site still hypersensitive to DNase I cleavage at –50 on the antisense strand regardless of the presence of inducer(s). Protection of these hypersensitive sites has been observed under conditions associated with activation of *benA* expression. Similarly, sites on the sense strand in the Site 2 vicinity failed to be protected by BenM in this mutant. In addition, BenM protection of Site 3 on the *benMA 5438* promoter does not appear to change significantly in the presence of inducer, suggesting that in the absence of Site 2, BenM binds strongly to Sites 1 and 3 regardless of the presence of inducer.

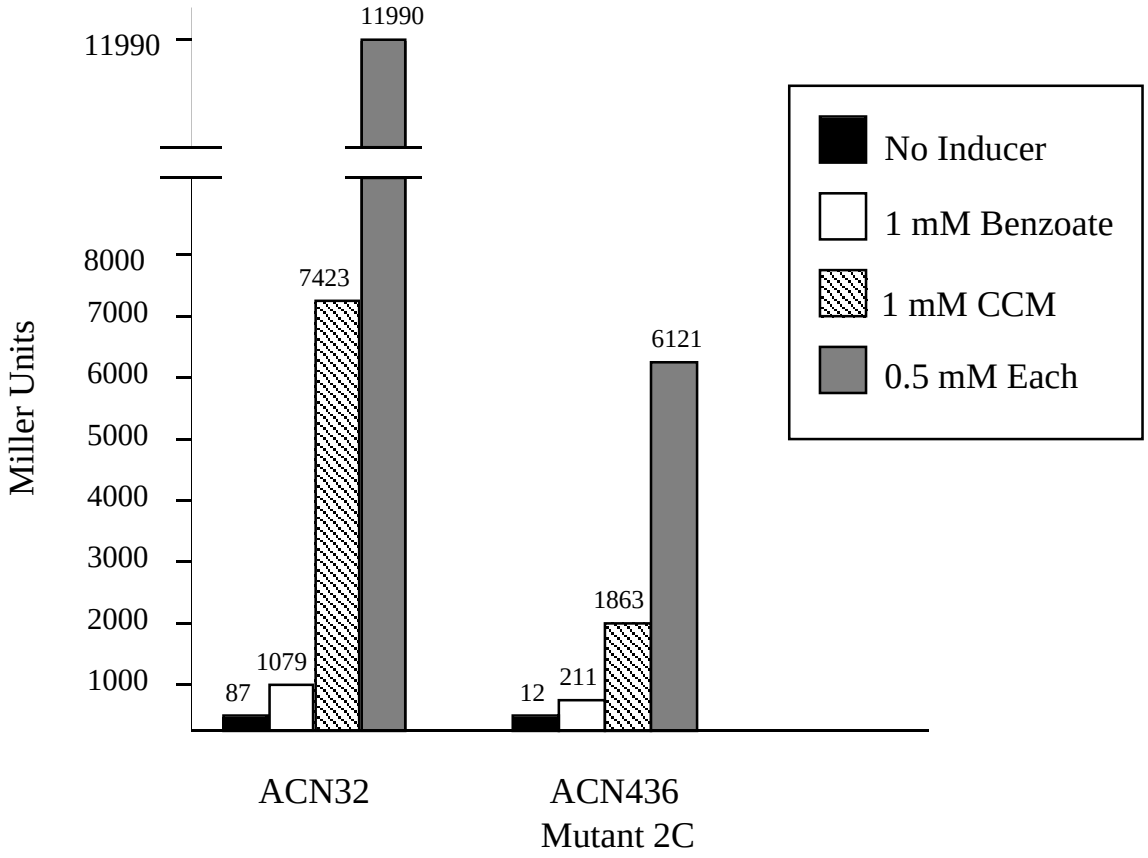


Figure 3.6. β-Galactosidase (*lacZ*) Analysis of a *benA::lacZ* Transcriptional Fusion. *In vivo* LacZ assay results of the wild-type and *benMA 5439* promoter mutation using a chromosomal *benA::lacZ* transcriptional fusion. Strains were grown in LB with no inducer, 1 mM benzoate, 1 mM CCM, or 0.5 mM each inducer as indicated. Results are reported as Miller units. Results are the average of 3 separate assays and Miller units are noted above each respective bar. The pair of horizontal lines indicates that the scale of the Y-axis has been interrupted.

In order to determine the effects of this Site 2 5' half site mutation *in vivo*, I constructed strain ACN435 in which the *benMA 5438* allele controls expression of a chromosomal *benA::lacZ* transcriptional fusion. β-galactosidase analysis of ACN435 as compared to ACN32, with a wild-type promoter, demonstrated that the *benMA 5*438 allele eliminates BenM-mediated *benA* expression (data not shown). Consistent with the failure of this Site 2 mutation to allow activation of *ben* gene expression, strain ACN438 with the *benMA 5438* chromosomal allele was unable to grow with benzoate as the sole carbon source.

DNase I footprinting of BenM at the *benMA 5440* promoter is shown in Figure 3.4D and 3.5D, on the antisense and sense strands, respectively. Mutation of both elements of dyad symmetry of Site 2 resulted in the loss of protection of Site 2 in response to both benzoate and CCM. In fact, BenM contacts at the *benMA 5440* promoter are nearly identical to that of BenM on the *benMA 5438* promoter (Fig. 3.4C and D and Fig. 3.5C and D). Evidence for loss of Site 2 protection on the *benMA 5440* promoter stems from the site still hypersensitive to DNase I cleavage at –50 on the antisense strand regardless of the presence of inducer(s). Similarly, sites on both the sense and antisense strands in the vicinity of Site 2 failed to be protected by BenM in this mutant. BenM protection of Site 3 does not change significantly in the presence of inducer on the *benMA 5440* promoter, suggesting that in the absence of Site 2, BenM binds strongly to Sites 1 and 3.

In order to determine the effects of this *benMA 5440* mutant allele *in vivo*, I constructed strain ACN460 in which the *benMA 5440* allele controls expression of a chromosomal *benA::lacZ* transcriptional fusion. β-galactosidase analysis of ACN460 as

compared to ACN32, with a wild-type promoter, demonstrated that the *benMA 5*440 allele eliminates BenM-mediated *benA* expression (Data not shown). Consistent with the failure of this Site 2 mutation to allow activation of *ben* gene expression, strain ACN440 with the *benMA 5438* chromosomal allele was unable to grow with benzoate as the sole carbon source.

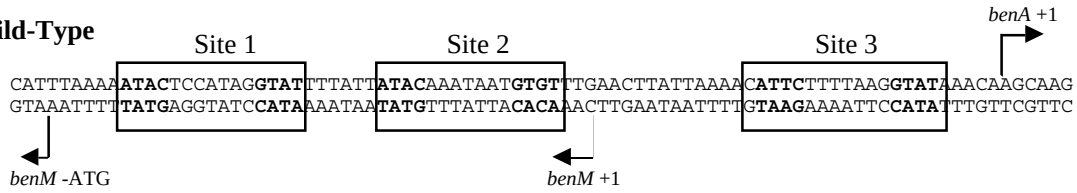
Analysis of 5 and 11 bp Insertions Between BenM-Binding Sites 1 and 2

In order to determine the importance of the spacing between the LTTR-binding sites of the *benA* promoter for BenM-mediated activation, 5 and 11 bp insertions were constructed between Sites 1 and 2. These insertion mutations, constructed in the same manner as the promoter mutations already described, are detailed in Figure 3.7. Allelic designations with strain and plasmid numbers are given in Table 3.1.

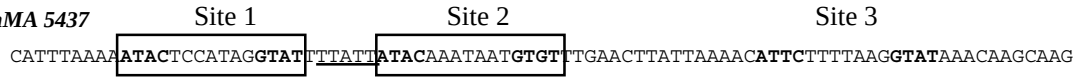
In allele *benMA 5437*, Insert 5-12, 5 bp were inserted between Sites 1 and 2. DNase I footprinting with BenM at the *benMA 5437* promoter is displayed in Figure 3.8B and 3.9B, on the antisense and sense strands, respectively. Insertion of 5 bp between Sites 1 and 2 should place both LTTR-binding Sites 2 and 3 on the opposite face of the DNA helix from the Site1, to which BenM binds most strongly. In the absense of inducer, there are marked differences in the way BenM protects the wild-type and *benMA5437* promoters from DNase I cleavage (Fig. 3.8 and 3.9 A and B, lanes 2). On the mutant promoter there is slight protection of Site 1, and perhaps some slight protection of Site 3. However, the strong protection of Sites 1 and 3, and the sites of hypersensitivity near Site 2 that are characteristic of the wild-type promoter are not observed on the mutant promoter.

Figure 3.7. Sequences of the Site Directed Insertion Mutations Created Between Sites 1 and 2, and between Sites 2 and 3. The DNA sequence of the wild-type *benA* promoter and the site-directed *benMA* promoter spacing mutations are given. Sites 1, 2, and 3 are indicated by boxes on the wild-type sequence, and by bold type on the mutant promoter sequences. The divergently transcribed *benA* and *benM* transcriptional start sites, designated +1 on each strand, respectively, are indicated by arrows on the wild-type sequence. The *benM* ATG translational start codon is also indicated with an arrow on the wild-type sequence. The Sites affected by insertions are indicated by solid boxes for each mutant, and the sequence of the resultant promoter is displayed below. Insertion mutants 5-12 and 5-23, which contain 5 extra base pairs between Sites 1 and 2, and Sites 2 and 3, respectively, are the result of a 5 bp duplication of the sequence TTATT which occurs between both sets of LTTR binding sites. Insertion mutants 11-12 and 11-23, which contain 11 extra base pairs between Sites 1 and 2, and Sites 2 and 3, respectively, are the result of the same 5 bp duplication described above separated by an additional 6 bp encoding the *Eco*RI restriction site. Duplicated regions are indicated by underscoring.

Wild-Type



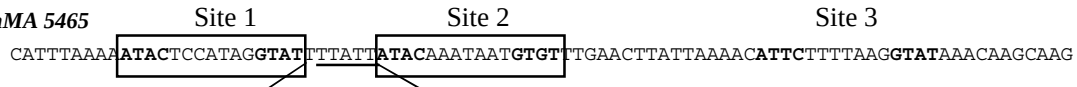
Insert 5-12
benMA 5437



TTATTTTATT

5 bp duplication between Sites 1 and 2

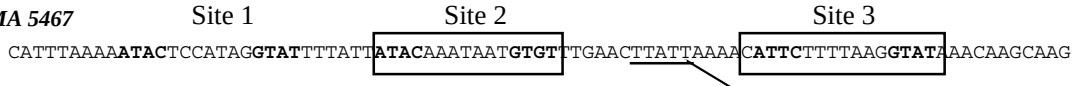
Insert 11-12
benMA 5465



TTATTGAATTCTTATT

*Eco*RI site added between duplication
(results in 11 bp insertion)

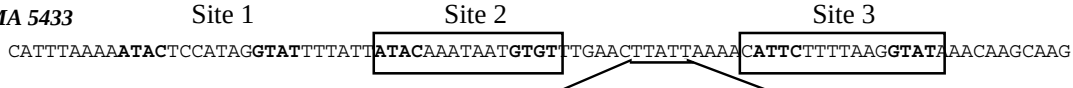
Insert 5-23
benMA 5467



5 bp duplication between Sites 2 and 3

TTATTTTATT

Insert 11-23
benMA 5433



*Eco*RI site added between duplication
(results in 11 bp insertion)

TTATTGAATTCTTATT

Figure 3.8. Analysis of the Ability of BenM to Bind to the LTTR-Binding Site Spacing Mutations on the Antisense Strand. Antisense-strand DNase I Footprinting analysis of the (A) wild-type; (B and C) 5 bp insertions between Sites 1 and 2, and Sites 2 and 3, respectively; and (D and E) 11 bp insertions between Sites 1 and 2, and Sites 2 and 3, respectively, as described in Fig. 3.6. Lanes 1 contain no BenM-His. Lanes 2 contain 3 pmol BenM-His with no inducer. Lanes 3 contain 3 pmol BenM-His with 1 mM benzoate. Lanes 4 contain 3 pmol BenM-His with 1 mM CCM. Lanes 5 contain 3 pmol BenM-His and 0.5 mM each benzoate and CCM. The approximate location of BenM-binding Sites 1, 2, and 3 are indicated to the right. The corresponding *benMA* allele and the sequence of the mutant region are shown below.

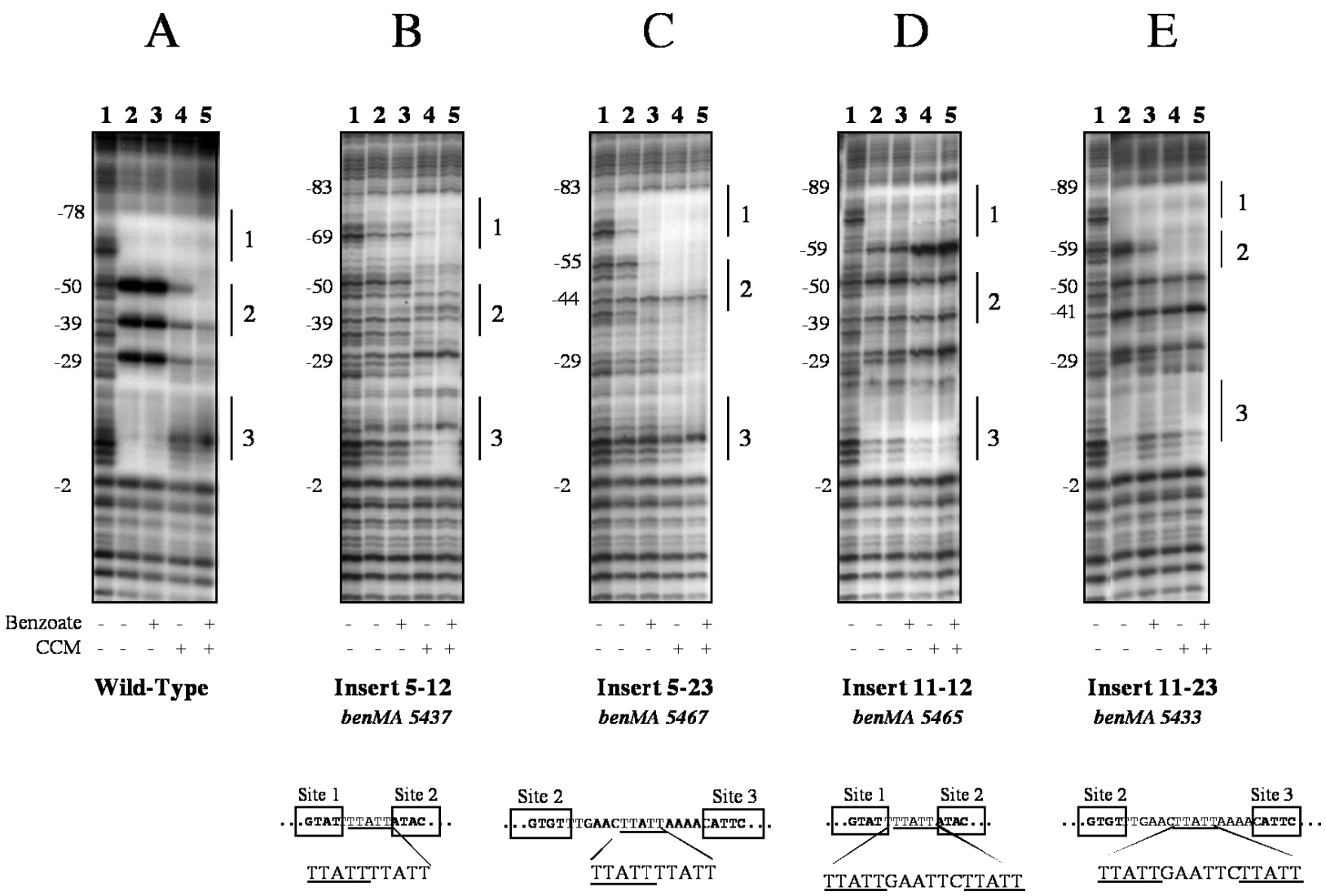
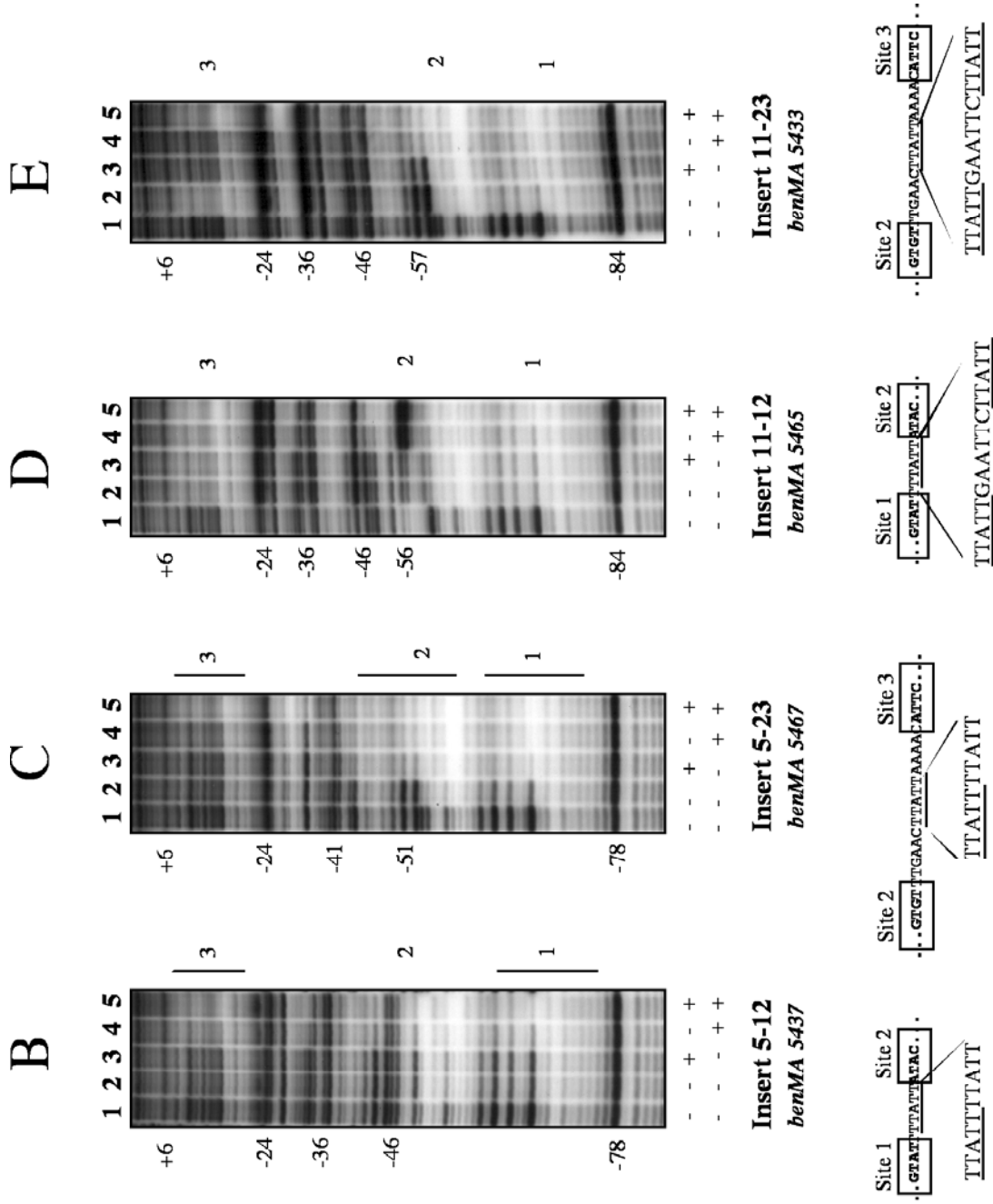

A
B
C
D
E
1 2 3 4 5
-78
-50
-39
-29
-2
-83
-69
-55
-44
-89
-59
-41
1
2
3
Benzoate - - + - +
CCM - - - + +
Wild-Type
Insert 5-12
benMA 5437
Insert 5-23
benMA 5467
Insert 11-12
benMA 5465
Insert 11-23
benMA 5433
Site 1
Site 2
Site 3
..GTATTTTATTATAC..
..GTGTTTGAACTTATTAAAACATTC..
TTATTTTATT
TTATTGAATTCTTATT

Figure 3.9. Analysis of the Ability of BenM to Bind to the LTTR-Binding Site Spacing Mutations on the Sense Strand. DNase I Footprinting analysis of the (A) wild-type; (B and C) 5 bp insertions between Sites 1 and 2, and Sites 2 and 3, respectively; and (D and E) 11 bp insertions between Sites 1 and 2, and Sites 2 and 3, respectively, as described in Fig. 3.6. Lanes 1 contain no BenM-His. Lanes 2 contain 3 pmol BenM-His with no inducer. Lanes 3 contain 3 pmol BenM-His with 1 mM benzoate. Lanes 4 contain 3 pmol BenM-His with 1 mM CCM. Lanes 5 contain 3 pmol BenM-His and 0.5 mM each benzoate and CCM. The approximate location of BenM-binding Sites 1, 2, and 3 are indicated to the right. The corresponding *benMA* allele and the sequence of the mutant region are shown below.


A
B
C
D
E
1 2 3 4 5
Benzoate
CCM
Wild-Type
Insert 5-12
benMA 5437
Insert 5-23
benMA 5467
Insert 11-12
benMA 5465
Insert 11-23
benMA 5433
Site 1
Site 2
Site 3
TTATTTTATT
TTATTGAATTCTTATT

These results suggest that BenM binding simultaneously to Sites 1 and 3, which normally causes looping-out of the intervening DNA and represses *benA* expression, is hindered by the addition of 5 bp between Sites 1 and 2. BenM tetramer binding to Site 1 may be stabilized by simultaneous binding to Site 2 or 3, depending on the presence of inducer, and so BenM protection of Site 1 may be weakened when it is unable to bind cooperatively to either of the other two sites.

The addition of CCM altered the BenM-protected regions of *benMA 5437*. The presence of CCM or both CCM and benzoate increased the protection of Sites 1 and 3 and also caused hypersensitivity to cleavage of a site near –29 (Fig. 3.8 and 3.9 A and B, Lanes 5). It appears the conformational changes in response to inducer increase the ability of BenM to bind to Sites 1 and 3. Although the mutation alters the helical face on which the protein would be expected to bind, the amount of DNA (approximately 5.5 helical turns) between these sites may allow enough flexibility for a tetramer of BenM to be able to contact both sites simultaneously. The altered pattern of hypersensitivity between the mutant and the wild-type (Fig. 3.8 A and B, Lanes 5) suggests that there are differences in the conformation of the intervening DNA between Sites 1 and 3 of these two promoters. Protection of the cleavage site at –50 on the antisense strand is observed in both the wild-type and mutant promoters (Fig. 3.8 A and B, Lanes 5), whereas on the sense strand the strong cleavage sites near –46 are protected on the wild-type but not on the *benMA 5437* promoter fragment (Fig. 3.9 A and B, lanes 5). While protection of these sites has been associated with high-level *ben* gene expression in the wild-type strain, the majority of the Site 2 region is not protected on the mutant promoter.

Therefore, the implications of these protection patterns for gene expression were not evident.

To investigate the effects of the 5 bp insertion between Sites 1 and 2 *in vivo*, I constructed strain ACN434 with the *benMA 5437* allele controlling *benA* expression of a chromosomal *benA::lacZ* transcriptional fusion. β-galactosidase analysis of ACN434, as compared to ACN32 which has a wild-type promoter, demonstrated no activation of *benA* expression. There was approximately 4-fold derepression of basal *ben* gene expression, consistent with poor protection of the *benMA 5437* Site 3 by BenM (Data not shown). Consistent with the failure of this promoter to allow strong BenM-Site 2 contacts to allow activation of *ben* gene expression, strain ACN437 with the *benMA 5437* chromosomal allele in an otherwise wild-type strain was unable to grow with benzoate as the sole carbon source.

In order to investigate spacing requirements of BenM at LTTR-binding Sites 1 and 2 further, I constructed Insert 11-12, designated allele *benMA 5465*. DNase I footprinting analysis of BenM at the *benMA 5465* promoter is displayed in Figure 3.8D and 3.9D, on the antisense and sense strands, respectively. Insertion of 11 bp between Sites 1 and 2 should conserve the positioning of the LTTR binding sites on the same face of the helix, while inserting a full helical turn of the DNA between Sites 1 and 2. As shown in figures 3.8D and 3.9D, as compared to the wild-type promoter (Fig. 3.8A and 3.9A), there is protection of LTTR-binding Sites 1 and 3 regardless of the presence of inducer. The affinity of BenM for Sites 1 and 3 on the *benMA 5465* allele appears weaker than that on the wild-type promoter, and the extent of the hypersensitivity of sites at 10 bp intervals between Sites 1 and 3 appears weaker overall. There is an extra site

hypersensitive to DNase I cleavage on the *benMA 5465* promoter, as compared to the wild-type, due to the insertion of a full helical turn between Sites 1 and 3.

There is no strong protection of Site 2 by BenM regardless of the presence of inducer. This is most evident looking at position –59, which corresponds to the wild-type position –50. This position is protected in the wild-type by BenM in the presence of CCM and benzoate together, but fails to be protected on the *benMA 5465* allele regardless of the presence of inducer. Since this mutant promoter contains a full helical turn between Sites 1 and 2, it appears that the spacing between these two sites is important for BenM-binding. The inability of a BenM tetramer to contact Site 2 may explain the protection of Site 3 regardless of the presence of inducer. If binding of BenM to the *benA* promoter is stabilized by cooperativity between BenM bound to Site 1 and either Site 2 or Site 3, BenM binding to Site 3 may be helping it to bind the *benA* promoter in the absence of Site 2-binding. This was also the case for the *benMA 5437* allele, where the presence of inducer appeared to increase the affinity of BenM for Sites 1 and 3 in the absence of benM-Site 2 contacts.

To investigate the effects of the 11 bp insertion between Sites 1 and 2 *in vivo*, I constructed strain ACN463 with the *benMA 5465* allele controlling *benA* expression of a chromosomal *benA::lacZ* transcriptional fusion. β-galactosidase analysis of ACN463, as compared to the ACN32 which has a wild-type promoter, demonstrated no activation of *benA* expression. Neither did ACN463 demonstrate derepression of basal *benA* expression, consistent with the relatively good binding of BenM to Site 3 regardless of the presence of inducer (Fig. 3.8 and 3.9 panel D) (data not shown). Consistent with the strong binding of BenM to Sites 1 and 3 at the *benMA 5465* promoter, strain ACN465,

with this allele controlling *ben* gene expression in an otherwise wild-type strain, failed to grow with benzoate as the sole carbon source.

Analysis of 5 and 11 bp Insertions Between BenM-Binding Sites 2 and 3

An additional mutation was constructed with 5 bp inserted between Sites 2 and 3, Insert 5-23 or allele *benMA 5467*. DNase I footprinting of the promoter with the 5 bp insertion between Sites 2 and 3, *benMA 5467*, was performed to investigate the effects on BenM binding to the *ben*A promoter. As shown in Figure 3.8C and 3.9C, there is no strong protection of Site 3 regardless of the presence of inducer, though some weak protection of Site 3 does appear to occur in the presence of CCM or both inducers. The absence of sites hypersensitive to DNase I cleavage support the fact that BenM does not appear to be binding Site 1 and Site 3 together. Most likely this is because the 5 bp insertion places these sites on opposite side of the DNA helix. BenM-binding to the *benMA 5467* Site 1 appears weaker than that of the wild-type (Fig. 3.8C and 3.9C as compared to 3.8A and 3.9A) in the absence of inducer. This weaker Site 1 association in the absence of inducer may be due to the loss of contacts with Site 3. If BenM contacts at the *benA* promoter are strengthened by the binding to Site 2 or 3 in addition to Site 1, then the loss of Site 3 binding may weaken BenM-Site1 contacts in the absence of inducer. Binding of BenM to Site 2 in the presence of inducer is evident even in the presence of benzoate alone, and may be due to the lessening of BenM-binding to Site 3. In order to investigate the effect of Insert 5-23 on *ben* gene expression *in vivo*, I constructed strain ACN464 with the *benA::lacZ* transcriptional fusion under the regulation of the *benMA 5467* allele. β-galactosidase analysis of ACN464, shows that

the insertion of 11 bp between Sites 2 and 3 only slightly lowers *benA* expression as compared to strain ACN32 ,with the wild-type promoter, in response to no inducer, either inducer alone, or both inducers together (Fig. 3.10). The active conformation of BenM may contact Sites 1 and 2 as usual on the *benMA 5467* promoter to induce *ben* gene expression. The slightly lower levels of *benA* expression may be due to the 5 bp inserted in the vicinity of the promoter regions recognized by RNAP. There is no derepression of basal *ben* gene expression, as might be expected for poor binding of BenM to Site 3, but this may also be due to the slight alteration of the –35 region. Consistent with the ability of BenM to contact Sites 1 and 2 on the *benMA 5467* promoter in a manner implicated to activate *benA* expression, strain ACN467 with this mutant promoter controlling *ben* gene expression in an otherwise wild-type strain grew on benzoate as the sole carbon source comparably to ADP1.

The final mutation to be discussed here is Insert 11-23, the *benMA 5433* allele, in which 11 bp were inserted between Sites 2 and 3. In this construct, Site 3 should be on the same face of the DNA helix as Sites 1 and 2. DNase I footprinting analysis of the *benMA 5433* promoter is displayed in Figure 3.8E and 3.9E, on the antisense and sense strands, respectively. BenM binding to Site 1 is strong on the *benMA 5433* allele regardless of the presence of inducer. In the absence of inducer, BenM binds Sites 1 and 3, and this protection of Site 3 lessens in the presence of either inducer. BenM-binding of Site 2 occurs in the presence of CCM or both inducers, as seen by the protection of the hypersensitive site at –59, which corresponds to the site at –50 in the wild-type (Fig. 3.8A and 3.9A).

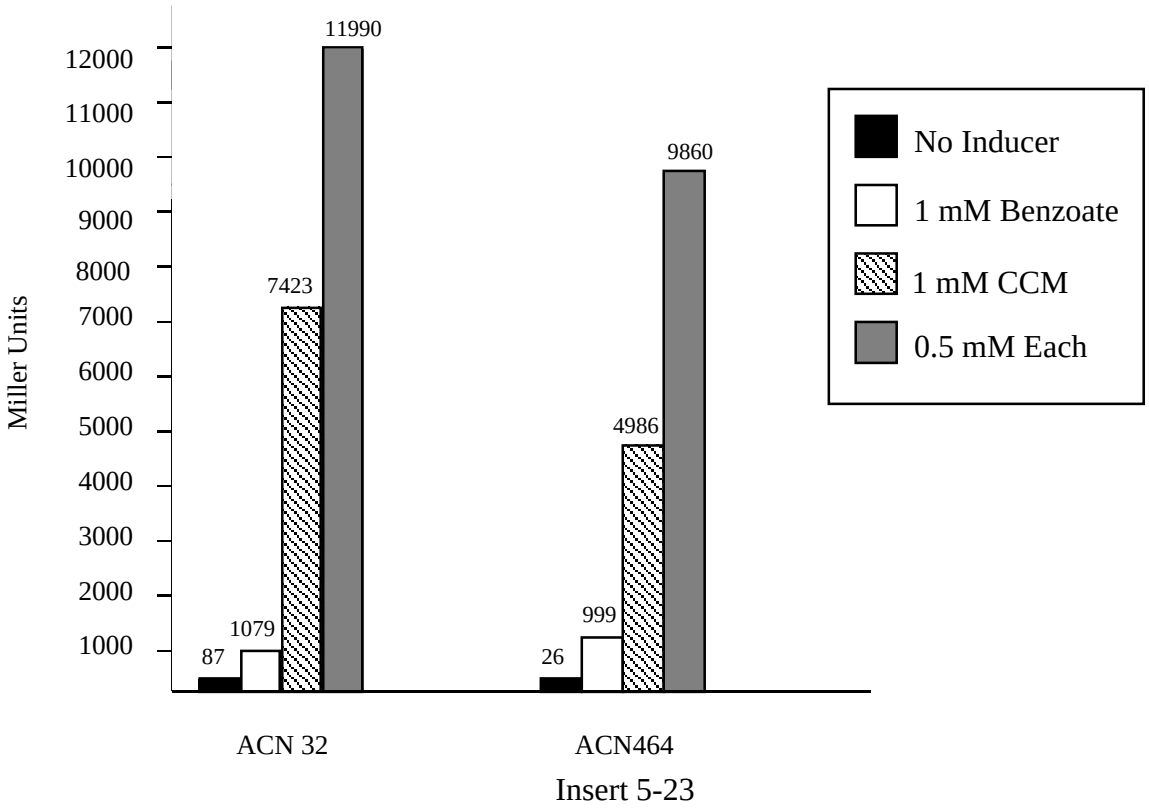


Figure 3.10. β-Galactosidase Assays of a *benA::lacZ* Transcriptional Fusion. *In vivo* LacZ assay results of the wild-type and *benMA 5467* promoter LTTR-binding site spacing mutation using a chromosomally encoded *benA::lacZ* transcriptional fusion. Strains were grown in LB with no inducer, 1 mM benzoate, 1 mM CCM, or 0.5 mM each inducer as indicated. Results are reported as Miller units. Results are the average of 3 separate assays and Miller units are noted above each respective bar.

The pattern of BenM-protection on the *benMA 5433* promoter is very similar to that on the wild-type promoter, except for the addition of an extra site sensitive to DNase I cleavage between Sites 1 and 3. This extra site is assumed to be due to the addition of an extra turn of the DNA helix, which allows for another site at 10 bp intervals to be cleaved in the looped-out region of the promoter DNA when BenM is bound to Sites 1 and 3.

In order to investigate the effect of Insert 11-23 on *ben* gene expression *in vivo*, I constructed strain ACN549 with the *benMA 5433* allele controlling expression of a *benA::lacZ* transcriptional fusion. β-galactosidase analysis of strain ACN459, as compared to ACN32 with the wild-type promoter, demonstrated that the insertion of 11 bp between Sites 2 and 3 eliminates *benA* expression (data not shown). There was no activation and no derepression of basal *ben* gene expression. From the observation that BenM contacts Sites 1 and 2 in the presence of CCM or both inducers on the Insert 11-23 promoter, it might be expected to be able to induce *ben* gene transcription *in vivo*. However, alteration of the spacing in the –35 promoter region of this allele may prevent transcription in this mutant. Consistent with the inability of the *benMA 5433* allele to activate transcription, strain ACN433 with the insertion of 11 bp between Sites 2 and 3 in an otherwise wild-type background was unable to grow on benzoate as the sole carbon source.

The studies presented in this chapter indicate that BenM-binding Site 1 may be necessary for cooperative binding to the non-consensus Sites 2 and 3. In fact, a similar cooperativity has been demonstrated for CatR of *P. putida* where binding of CatR to an upstream putative "recognition binding site" is required for CatR binding to a

downstream non-consensus "activation binding site" (Parsek et al., 1992). In addition, the studies presented in this chapter support the requirement of Site 2 for BenM-mediated inducer synergy, and also for activation of *ben* gene expression in response to either inducer alone. This result is consistent with our hypothesis that BenM may adopt two possible conformations with the *benA* promoter DNA, and that the presence of no inducer, either inducer, or both inducers together determines the equilibrium between these two BenM-DNA conformations. The BenM-DNA complex with protein bound to Sites 1 and 2 is the "active" conformation, and either inducer alone shifts the equilibrium towards this conformation. The presence of both benzoate and CCM shifts the equilibrium even further towards the active conformation, perhaps keeping the BenM-DNA complex more completely in this active state.

The studies presented in this chapter also support our hypothesis that the three possible BenM-binding sites in the *benA* promoter function optimally when they are located on the same side of the DNA helix. Insertion of 5 bp between Sites 1 and 2 resulted in the loss of BenM-mediated *ben* gene activation and resulted in inhibition of BenM-binding to Site 3. The insertion of 5 bp between Sites 2 and 3, though it only slightly inhibited *ben* gene expression, demonstrated that BenM no longer bound Site 3 well. The insertion of 5 bp between Site 1 and Site 3 therefore places Site 3 on the opposite side of the DNA helix and results in the loss of BenM-Site 3 contacts. Additionally, these studies demonstrated that the spacing between Sites 1 and 2 is important, as insertion of 5 or 11 bp between these sites resulted in the loss of BenM-Site 2 contacts. Taken together, these studies have increased our understanding of and helped strengthen our model of BenM-mediated *benA* expression.

Experimental Procedures

Bacterial Strains, DNA Isolation, and Growth Conditions

Escherichia coli DH5α (Gibco BRL) was used as a plasmid host. *E. coli* were cultured in Luria-Bertani (LB) broth, while *Acinetobacter* strains were grown in a minimal medium as previously described (Shanley et al., 1986). Unless indicated otherwise, 2 mM benzoate served as the sole carbon source for *Acinetobacter* strains. Antibiotics were added as needed at the following concentrations: tetracycline (Tc), 6 ug/ml; kanamycin (Km), 25 ug/ml; ampicillin (Ap), 150 ug/ml. Standard methods were used for plasmid purifications, restriction enzyme digestions, electrophoresis, ligations, and *E. coli* transformations (Sambrook et al., 1989).

Strain and Plasmid Construction

Plasmids carrying the *benMA* mutant alleles were constructed according to the Altered Sites II *in vitro* Mutagenesis System (Promega). To construct the site directed mutations, the 1.3 kbp *Kpn*I to *Pst*I wild-type *benMA* promoter fragment from pBAC14 (Collier et al., 1998) was cloned into the vector pALTER®-1. The resultant plasmid was designated pBAC477. Primers used to construct the site directed mutations, and the corresponding resultant plasmids, are listed in Table 3.1. The *benMA 5466* and *benMA 5467* alleles, pBAC522 and pBAC521, are in pUC19. These two site directed mutant alleles were difficult to separate from the parent plasmid, pBAC477, and so were amplified by PCR and cloned into pUC19. The primers used were of sequence 5'GATTAAGCGGATGGCTGGCAT3' and 5' ATACTGCAGGCTAGTATTAATGACGGGAAT3'. Resultant plasmids were screened

by restriction endonuclease digestion to check for the *Eco*RI sites added through mutagenesis, and confirmed by DNA sequence analysis. To determine if these mutations effected the growth of ADP1 on benzoate as a sole carbon source, the natural transformation system of ADP1 was utilized. The mutant *benMA* alleles were amplified by PCR using M13for and M13rev primers, and the resultant DNA fragments used to replace the corresponding region of the ADP1 chromosome in both a wild-type and *benA::lacZ* background. Resultant strains are listed in Table 3.1.

DNase I Footprinting

Footprinting experiments were carried out as described in Chapter 2. Plasmids used are listed in Table 3.1. Plasmids with the mutant *benMA* alleles suitable for antisense-strand footprinting experiments were constructed by PCR amplification of the mutant alleles using primers of sequence 5'GATTAAGCGGATGGCTGGCAT3' and 5'CGAATATCCATTTCAGCTTT3'. DNA fragmentsb were subsequently digested with *Kpn*I and *Nsi*I, and then cloned into puc19 digested with *Kpn*I and *Pst*I. Resultant plasmids are listed in Table 3.1. For sense strand footprinting plasmids, *Hin*dIII deletions of the corresponding antisense-strand footprinting plasmids were constructed. For Antisense strand footprinting, plasmids were initially digested with *Eco*RI or *Asp*718, end-labeled as described in Chapter 2, and the 3' label released by *Hin*dIII digestion. For sense-strand footprinting, plasmids were initially digested with *Hin*dIII, end-labeled as previously described, and the 3' label released by *Eco*RI digestion.

β-Galactosidase Assays

Cultures were grown overnight in 5 ml of LB with or without 2 mM benzoate, 2 mM cis,cis-muconate, or 1 mM of each inducer. Cells were lysed with chloroform and sodium dodecyl sulfate, and β-galactosidase activities were determined as described previously (Miller, 1972)

CHAPTER 4

CONCLUSIONS

The studies presented in this dissertation have contributed to our understanding of the complex regulatory circuits controlling aromatic compound catabolism in natural systems. We must first understand how to control predictably the expression of biodegradative genes before attempting to engineer these systems for biotechnological applications. Aromatic pathway regulatory proteins fall into a number of families including the NtrC, LysR, AraC, IclR, FNR, MarR, and GntR families. Most of these regulators for controlling aromatic compound degradation are transcriptional activators, with the exception of the GntR family, that interact with either the aromatic compound itself or a pathway intermediate to induce expression of the catabolic genes (Diaz and Prieto, 2000). The studies presented in this dissertation describe in detail for the first time a transcriptional regulator, BenM, that responds to the presence of both the aromatic compound (benzoate) and an intermediate of the pathway (CCM) to induce a synergistic level of degradative-gene expression.

In order to investigate this BenM-mediated synergy *in vitro*, we purified BenM and another closely related LTTR, CatM. *In vitro* transcription assays confirmed that benzoate and CCM are both, individually, inducers of BenM-mediated activation of the *ben* genes. These assays also confirmed that benzoate and CCM induce a synergistic level of BenM-mediated *ben* gene expression, and that no additional *Acinetobacter* proteins are required.

DNase I footprinting assays demonstrated that benzoate and CCM together altered the BenM-DNA promoter regulatory region interactions as compared to those with either inducer alone. Sequence gazing suggested that three sites exist in the *ben* gene promoter that BenM might be able to bind. A putative LTTR-binding site was centered at –64, with respect to the *benA* transcriptional start site, and named Site 1. Two similar but non-consensus sites were centered at -43 and –12, named Sites 2 and 3, respectively. In the absence of inducer, footprinting assays demonstrated that BenM binds Sites 1 and 3, looping-out the intervening DNA, and causing three sites of hypersensitivity in the region of Site 2. It is assumed that this DNA loop and the binding of BenM overlapping the RNAP promoter interferes with RNAP-binding, resulting in 2-fold repression of the *ben* genes. When benzoate or CCM are present in the footprinting reactions, BenM no longer strongly binds to Site 3, allowing a small amount of *ben* gene transcription by RNAP. When both benzoate and CCM are present BenM assumes a conformation that allows it to bind Sites 1 and 2, resulting in full transcriptional activation of the *ben* genes.

Full induction of the *ben* genes only occurs in the presence of both inducers. This may be due to the fact that in the presence of either inducer alone there is still rather weak association of BenM with Site 3 that keeps the level of RNAP association with the *benA* promoter low. It is also likely that full *ben* gene induction only occurs in the presence of both inducers because they are both required to lock BenM in a conformation that binds strongly to Sites 1 and 2. Site 2 is in a position typical of the "activation binding site" of most LTTRs of the CCM- and chloro-CCM binding subclass. This position is upstream of and overlapping the RNAP -35 promoter regulatory region and is responsible for enabling the LTTR-RNAP αCTD interactions that have been demonstrated to account for

induction in a number of LTTR-regulated systems (Tao et al., 1993; Gussin et al., 1992; Shi and Bennett, 1994; Chugani et al., 1997; McFall et al., 1997).

Mutational analysis of the *benABCDE* promoter region, detailed in Chapter 3, confirmed our model of BenM-mediated *ben* gene expression. *In vivo* and *in vitro* assays demonstrated that Site 1 is required for BenM binding to the *benA* promoter region, and is therefore required for high-level *ben* gene expression. These assays also demonstrated that Site 2 is absolutely required for BenM-mediated synergy and full induction of the *ben* genes. A previously identified –8 constitutive promoter mutation (Collier et al., 1998) was also used to confirm that BenM binding to Site 3 results in repression of the *ben* genes. Comparison of the wild-type and –8 constitutive promoters demonstrated that the –8 mutation may slightly weaken the BenM-Site 3 interaction. This –8 mutation also increases RNAP-promoter interactions that result in higher basal *ben* gene expression as evidenced by *in vitro* transcription assays where in the absence of BenM the constitutive promoter led to high levels of benA transcription. It induces *ben* gene expression to levels high enough for growth on benzoate as a sole carbon and energy source.

CatM is 59% identical to BenM and regulates expression of the *catA* and *catBCIJFD* operons in response to CCM. CatM does not activate *ben* gene expression *in vivo* even though it binds the *ben* promoter region regardless of the presence of inducer. DNase I footprinting assays demonstrated that CatM binding at the *ben* promoter is nearly identical to that of BenM. In addition, the response of CatM to the presence of CCM in the footprinting assays is nearly indistinguishable from BenM in response to CCM. *In vivo* studies suggested that CatM, unlike BenM, does not respond to benzoate. *In vitro* transcription and DNase I footprinting assays confirmed that CatM does not

respond to benzoate as an inducer compound. DNase I footprinting assays, described in Chapter 3, demonstrated that a single point mutation at –40 in Site 2 enables CatM in the presence of CCM to contact the *benA* promoter in a way analogous to that of BenM in response to benzoate and CCM. This point mutation allows CatM to bind Sites 1 and 2 in response to CCM alone, resulting in transcriptional activation of the *ben* genes to a level high enough to support growth on benzoate.

It is possible that when ADP1 is grown on benzoate, BenM is able to respond to low intracellular benzoate levels to up-regulate *ben* gene expression. This would result in the production of some CCM, which could then act with benzoate to induce high-level *ben* gene expression. Since CatM is unable to respond to benzoate, and in fact continues to repress *ben*-gene expression the presence of benzoate, it is likely that CCM amounts in the cell never reach a level high enough to induce CatM-mediated *ben* gene expression. The –40 point mutation may therefore enable CatM to induce *ben* gene expression significantly at very low intracellular levels of CCM present in the cell due to the low basal level of *ben* gene expression.

These studies have greatly improved our understanding of BenM-mediated induction of the *ben* genes for benzoate degradation in *Acinetobacter* sp. strain ADP1. The ability of BenM to respond synergistically to two different inducers, both the target compound and an intermediate of its degradation, allows for an extremely sensitive fine-tuning mechanism in aromatic compound degradative pathway control. The results of this work may prove a new and useful tool for future biotechnological applications.

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