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The Complex *bet* Promoters of *Escherichia coli*: Regulation by Oxygen (ArcA), Choline (BetI), and Osmotic Stress

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The *bet* regulon allows *Escherichia coli* to synthesize the osmoprotectant glycine betaine from choline. It comprises a regulatory gene, *betI*, and three structural genes: *betT* (choline porter), *betA* (choline dehydrogenase), and *betB* (betaine aldehyde dehydrogenase). The *bet* genes are regulated by oxygen, choline, and osmotic stress. Primer extension analysis identified two partially overlapping promoters which were responsible for the divergent expression of the *betT* and *betIBA* transcripts. The transcripts were initiated 61 bp apart. Regulation of the promoters was investigated by using *cat* (chloramphenicol acetyltransferase) and *lacZ* (β-galactosidase) operon fusions. Mutation of *betI* on plasmid F′2 revealed that BetI is a repressor which regulates both promoters simultaneously in response to the inducer choline. Both promoters remained inducible by osmotic stress in a *betI* mutant background. On the basis of experiments with *hns* and *hns rpoS* mutants, we conclude that osmoregulation of the *bet* promoters was *hns* independent. The *bet* promoters were repressed by ArcA under anaerobic growth conditions. An 89-bp promoter fragment, as well as all larger fragments tested, which included both transcriptional start points, displayed osmotic induction and BetI-dependent choline regulation when linked with a *cat* reporter gene on plasmid pKK232-8. Flanking DNA, presumably on the *betT* side of the promoter region, appeared to be needed for ArcA-dependent regulation of both promoters.

Hyperosmotically stressed cells of *Escherichia coli* build up the cytoplasmic osmolarity by accumulation of potassium glutamate and various osmoprotectants (12). The highest osmotolerance is achieved by the accumulation of glycine betaine (hereafter called betaine) (49). Betaine can either be taken up by the ProU and ProP systems (7, 8, 37) or be synthesized by the Bet system, i.e., the choline-to-betaine pathway (31). Synthesis of betaine requires an external supply of choline or the intermediate metabolite betaine aldehyde. At low external concentrations, choline is mainly taken up by the high-affinity choline porter BetT (K_m = 8 μM), whereas at higher concentrations choline is also taken up by ProU (K_m = 1.5 mM) (30, 50). Oxygen-dependent choline dehydrogenase (BetA) catalyzes both steps in the oxidation of choline to betaine by the way of betaine aldehyde, whereas NAD-dependent betaine aldehyde dehydrogenase (BetB) is specific for the last step (1, 31).

Biochemical data have previously revealed that expression of the Bet system is reduced under anaerobic conditions. For aerobic cells, osmotic stress gives a partial induction, but for full expression, the cells also need an external supply of choline (31). Experiments with *lacZ* fusions showed that the regulation occurs at the level of transcription (16).

The DNA sequence of the *bet* region has revealed that in addition to the structural genes, the *bet* system encodes a regulatory protein called BetI. Albeit BetI shares some sequence homology with the TetR family of bacterial regulatory proteins (29), BetI seems to belong to a new type of repressor (see the accompanying paper [44]). The *bet* genes are tightly spaced within a region of 5.9 kb. Judging from the DNA sequence, *betIBA* constitutes one transcript whereas *betT* constitutes a separate transcript with divergent orientation. Because of the lack of mutations in *betI*, the exact function of BetI has not been studied previously. Apparently BetI takes part in the choline regulation of the *bet* regulon (29), but there are no data demonstrating whether BetI also participates in the osmoregulation of the *bet* gene expression.

ArcA, which is the regulatory protein of a two-component system, controls the activity of many *E. coli* genes which are repressed under anaerobic conditions (reviewed in reference 24). Fnr is another globular regulator, which controls the activity of many genes which are derepressed under anaerobic conditions. Fnr is required for full expression of *arcA* (10), and in some cases Fnr also directly participates in the regulation of ArcA-controlled genes (15).

There does not appear to be any universal mechanism of osmotic regulation of gene expression in *E. coli*. Regulation of the *kdpABC* operon (43) and regulation of the *ompF* and *ompC* genes are mediated by two-component systems (reference 40 and references therein). For other genes, no regulatory proteins of the typical repressor or activator type have been shown to be responsible for the osmotic regulation. Expression of several osmotically inducible genes depends on the stationary-phase-induced σ^S factor (RpoS), which is induced by osmotic stress at the level of translation (3, 33). The basal level of σ^S translation is increased in an *hns* (*osmZ*) mutant background (3, 53). Also, the basal level of *proU* expression is increased in an *hns* mutant background (21). However, as with *bet*, the expression of the major *proU* promoter is σ^S independent (25).

The *proU* and *bet* systems differ in that the degree of osmotic induction of *proU* is much higher than for *bet* and that *proU* is regulated only by osmotic stress. In this investigation we demonstrate that regulation of *bet* by choline, oxygen, and osmotic stress is mediated by three separate mechanisms; i.e., BetI, ArcA, and an as yet unidentified osmotic signal. In an accompanying paper, we report on the in vitro binding of BetI to the *bet* promoter region (44).

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TABLE 1. *E. coli* strains used in this study

Strain	Description[a]	Construction, source, or reference[b]
DH5αF$^-$	(F$^-$) ϕ80d *lacZ*ΔM15 *recA1 endA1 hsdR17 supE44 thi-1 gyrA96*λ$^-$ *relA1* Δ(*argF-lac*)*U169*	BRL
DH5αF′	(F′) DH5αF$^-$	BRL
FC2	MC4100 Δ*fnr*	11
FF33	[F′2 (*betT*$^+$ *betA5-lacZ* Kn^r)] MC4100	This study
FF914	[F′2 (*betT4-lacZ betIBA*$^+$ Kn^r)] MC4100	This study
FF2005	MC4100 Δ(*srl-300*::Tn*10*) *recA56*	Laboratory strain
GM161	*thr-1 leuB6 dam-4 thi-1 hsd51 lacY1 tonA21* λ$^-$ *supE44*	CGSC 6476
MC4100	(F$^-$) *araD139* Δ(*argF-lac*)*U169 flbB5301 relA1 rpsL150 deoC1 ptsF25 rbsR*	CGSC 6152
MLE33	[F′2 (*betT*$^+$ *betA5-lacZ* Kn^r)] MC4100 *recA56* Rif^r Thi$^-$	16
MLE914	[F′2 (*betT4-lacZ betIBA*$^+$ Kn^r)] MC4100 *recA56* Rif^r Thi$^-$	16
PD32	MC4100 *hns-206*::Ap^r	14
QC1993	[F (*sodA-lacZ)1*] GC4468 Δ*sodA3* λ Δ*arcA*::Tc^r *zjj*::mini-kan *zdh*::mini-kan-omega Sp^r Tc^r (6 μg/ml) Sm^r	I. Compan
RH90	MC4100 *rpoS359*::Tn*10*	32
SH205	HfrC *pho8 glpD3 glpR2 relA1 tonA22* (λ) Δ(*argF-lac*)*U169 zah-735*::Tn*10*	46
TL722	[F′2 (*betT*$^+$ *betIBA*$^+$)] MC4100	30
TT42	[F′2 (*betT4-lacZ betIBA*$^+$ Kn^r)] V355 Δ(*argF-lac*)*U169*	This study
TT44	[F′2 (*betT4-lacZ betI1*::*cat* Δ*betBA* Kn^r Tc^r)] MC4100	This study
TT50	MC4100 Δ*arcA*::Tc^r	P1(QC1993) × MC4100
TT51	[F′2 (*betT4-lacZ betIBA*$^+$ Kn^r)] TT50	TT50 × MLE914
TT52	[F′2 (*betT*$^+$ *betA5-lacZ* Kn^r)] TT50	TT50 × MLE33
TT53	[F′2 (*betT4-lacZ betIBA*$^+$ Kn^r)] FC2	This study
TT54	[F′2 (*betT*$^+$ *betA5-lacZ* Kn^r)] FC2	This study
TT64	TT44 *hns-206*::Ap^r	P1 (PD32) × TT44
TT68	[F′2 (*betT4-lacZ betI1*::*cat* Δ*betBA* Kn^r Tc^r)] RH90	This study
TT69	TT68 *hns-206*::Ap^r	P1(PD32) × TT68
V355	*lac-3350 galK2 galT22* λ$^-$ *recD1014*(Nuc$^-$) *rpsL179* IN(*rrnD-rrnE*)*1*	CGSC 6720 (9)

[a] *bet-lacZ* indicates that the gene contains a λ *plac*Mu53 (Kn^r) insertion which was generated in vivo (16). Rif^r, rifampin resistance.
[b] BRL, Bethesda Research Laboratories. CGSC, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn. All CGSC strains were obtained from B. J. Bachmann.

MATERIALS AND METHODS

Growth conditions. The minimal growth media used were low osmolarity medium (LOM) (8) and medium 63 (39) supplemented with 10 and 20 mM glucose, respectively. The osmotic strength was increased by the addition of 0.3 M NaCl. Choline (1 mM) was added as indicated in Results. The antibiotics used were ampicillin (100 μg ml^{-1}), tetracycline (15 μg ml^{-1}), chloramphenicol (30 μg ml^{-1}), streptomycin (100 μg ml^{-1}), and kanamycin (60 μg ml^{-1}). Cells were grown at 37°C.

For assaying chloramphenicol acetyltransferase (CAT) and β-galactosidase activities, cells were grown overnight in LOM or medium 63. Fresh overnight cultures were diluted in the same medium, and the growth was continued for 2 h. NaCl and choline were then added as indicated in the text, and the growth was continued for 3 h up to an A_{600} of 0.5 to 1.0. Anaerobic cultures were grown to an A_{600} of 0.5 to 1.0 in medium 63 for more than 10 h in flasks sealed with rubber stoppers and flushed with nitrogen.

Bacterial strains. The bacterial strains used are listed in Table 1. All strains used to measure *bet* promoter activities were derived from MC4100 and carried the (*argF-lac*)*U169* deletion which encompasses the *bet* genes. Transduction with P1 and conjugation were performed as described by Miller (39). Because *hns* mutants are known to accumulate second-site mutations (3), experiments with cells carrying *hns*::Ap^r (ampicillin resistance) were always performed with newly constructed strains. Strain GM161 was used for isolation of plasmids without Dam methylation.

Selecting for Kn^r (kanamycin resistance) and Ap^r, the plasmids F′2 (Kn^r) of MLE33 and MLE914 carrying *bet-lacZ* fusions (16) were conjugated into strain DH5αF$^-$ (Sm^s) carrying pGEM-3Zf(+) (Ap^r; Promega). From this strain and selecting for Kn^r and Sm^r (streptomycin resistance), the plasmids F′2 were further conjugated into MC4100, creating FF33 and FF914, and into FC2, creating TT54 and TT53. For construction of strain TT42, Δ(*argF-lac*)*U169* and the adjacent *zah*::Tn*10* of strain SH205 were transduced into strain V355. Then F′2 of MLE914 was inserted by conjugation, selecting for Kn^r and Tc^r (tetracycline resistance); this was followed by deletion of Tn*10* by the method of Bochner et al. (4). For construction of strain TT68, plasmid F′2 of TT44 was conjugated via DH5αF$^-$(pGEM-3Zf[+]), selecting for Kn^r and Ap^r, and further into strain RH90, selecting for Kn^r and Sm^r.

Recombinant DNA procedures. Isolation of plasmid DNA, cloning, and electrophoresis of DNA in agarose were done essentially as described by Maniatis et al. (35). Individual restriction fragments were isolated from agarose gels by centrifugation through siliconized glass wool (19). High-frequency transformation of ligated DNA was performed as described by Inoue et al. (22), using DH5αF′ as the recipient.

Plasmid constructions. Several DNA fragments from the *bet* region were inserted into the polylinker of vector pKK232-8 (6). Except for the fragment of plasmid pIB274 (described in Results), the extent and orientation of all these *bet* fragments are shown in Fig. 1. The 173-bp *bet* fragment of plasmids pTB173 and pIB173 was cloned as a PCR product, which was generated as described by Røkenes et al. (44). All other *bet* fragments were subcloned as restriction fragments, which were derived from plasmid pFF221 (1). Fragments were made blunt by Klenow polymerase if they were not compatible with target sites. Plasmid pAB4764 was made by insertion of a *Bam*HI linker (GGGATCCC) (Boehringer Mannheim) into the *Cla*I site within the *betI* gene of pAB4754. This resulted in a defective *betI* gene.

An *Eco*RI-*Bgl*II fragment (*bet* coordinates 2096 to 4257) was cloned into M13, and an artificial *Eco*RI site was created 11 bp upstream of the BetI coding region by in vitro mutagenesis by the method of Su and El-Gewely (51). The mutagenesis changed the original sequence (coordinates 2484 to 2493) from TGGAGT GGCG to TGGAATTCCG (the new *Eco*RI site is underlined). The resulting mutagenesis was verified by DNA sequencing. The new *Eco*RI site was used to construct plasmids pIB89 and pTB89 (see Fig. 1) and plasmid pFF440, which was used in the studies described in the accompanying paper (44). pFF440 contains a 1.8-kb *Eco*RI-*Xba*I fragment (encompassing *bet* coordinates 2486 to 4257) cloned into vector pGEM-7Zf(−).

Plasmid pTP100 was made by inserting an *Eco*RI-*Bgl*II fragment (coordinates 2096 to 4257) from plasmid pFF221 (1) into the *Eco*RI-*Hin*cII sites of vector pJRD184 (20). Control plasmid pTP101 was made by insertion of a 0.1-kb *Hin*cII-*Eco*RI fragment from the polylinker of pUC19 (Pharmacia) into the corresponding sites of pJRD184. For construction of plasmid pTP200, the *Eco*RI-*Bgl*II fragment of pFF221 was cloned into the *Eco*RI-*Bam*HI sites of pJRD184; this was followed by an insertion of the transcriptional terminator T1 from pKK232-8 (a 180-bp *Eco*RI fragment) into the *Eco*RI site of the resulting plasmid.

Insertion of *betI*::*cat* on plasmid F′2. Plasmid pTL300 was constructed in order to insert a *betI*::*cat* fusion into the *bet* genes on F′2. A 1.8-kb *Kpn*I-*Bam*HI fragment containing the 3′ end of *betA* and its downstream region was isolated from plasmid pFF221 and ligated into the polylinker of pGEM-7Zf(+). A 1.3-kb *Pvu*II fragment containing the 3′ end of the *cat* gene was purified from pKK232-8 and ligated into the *Eco*RV site of the chromosomal fragment in the same orientation as the *betA* gene. The *Eco*RV site was located downstream of *betA* and 1.3-kb from *Bam*HI. The resulting plasmid was linearized with *Eco*RI, which cut in the polylinker of the vector and in the *cat* gene, and a 0.9-kb *Eco*RI fragment containing the 5′ end of the *betI*::*cat* fusion of pIB2861 (see Fig. 1) was inserted. This created a plasmid insert containing a complete *betI*::*cat* fusion

starting with a *bet* fragment of 625 bp and ending with a 1.3-kb *Eco*RV-*Bam*HI fragment from the chromosomal region downstream of *betA*. We have found by DNA sequencing that this 1.3-kb fragment contains a Dam-methylated *Cla*I site 3 bp downstream of the *Eco*RV site. To make plasmid pTL300, a 2.1-kb *Eco*RI-*Pvu*II fragment containing the Tc^r marker of pBR322 was cloned into this *Cla*I site.

The 5.5-kb insert of pTL300 (see Fig. 3) was excised with *Xba*I and *Nsi*I and inserted into plasmid F′2 (*betT-lacZ*) of TT42 (Δ*bet recD*) by transformation, as described previously for *recD*-containing strains (45). F′2 of TT42 confers a Bet^+ phenotype (protection against osmotic stress by choline) on the chromosomal strain from which *bet* has been deleted, because the ProU system can conduct low-affinity uptake of choline (30). Mutants of strain TT42, in which the two resistance markers of the 5.5-kb fragment are integrated in the *bet* region on F′2, will have a Bet^- phenotype because of a deletion of the *betBA* genes, as illustrated in Fig. 3. Therefore, we selected for Tc^r transformants and scored for a Cm^r (chloramphenicol resistance) Kn^r Bet^- phenotype. A P1 lysate was grown on a transformant with the expected phenotype and used to transduce strain TL722, which carried a native plasmid F′2 (*betIBA*$^+$ *betT*$^+$), to Kn^r. Of 30 Kn^r transductants tested, 24 carried the three markers Tc^r, Cm^r, and Bet^- (e.g., TT44; Table 1), whereas the remaining six were Tc^s Cm^s Bet^+. These data showed that Kn^r (*betT-lacZ*), *betI*::*cat*, and Tc^r are linked on F′2. As would be expected for a construct in which the *cat* gene is under the control of the *betI* promoter, the Cm^r conferred by strains carrying this fusion was rather weak. Therefore, to prevent selection of *betI* promoter mutations which increased the CAT activity, Cm^r was never used as a selective marker.

To verify the structure of the mutant F′2, whole-cell DNA was isolated from strains TT44 (mutant F′2) and TT42 (negative control) and digested with *Bam*HI, *Bam*HI-*Eco*RI, or *Eco*RV. Southern blot analysis was performed according to the DIG System (digoxigenin) user's guide for filter hybridization (Boehringer Mannheim). Size markers were corresponding digests of the plasmid pTL300, which also was used as the probe.

Primer extension analysis. Total cellular mRNA isolation and primer extension reactions were performed essentially as described by Ausubel et al. (2), making use of single-stranded DNA primers (Biotechnology Centre of Oslo) end-labelled with [γ-^{32}P]dATP (Amersham) and T_4 polynucleotide kinase (Amersham). The DNA primers used were CGAAGCTCGGCGGATTTGTCC TACTCAAGC and CGATGCGATTGGGATATATCAACGGTGG, which had their 3′ ends within the polylinker and the *cat* gene, respectively, of vector pKK232-8. Radiolabelled primers were purified on S-300 microspin columns (Pharmacia). After hybridization with RNA and extension with reverse transcriptase, the products were separated on a sequencing gel and sized by comparison with sequence ladders derived from the same DNA primers. DNA sequencing was performed with the Sequenase version 2.0 sequencing kit (United States Biochemicals).

Enzyme assays. For CAT assays, cell suspensions were harvested, washed, and 10-fold concentrated in 0.1 M Tris-HCl (pH 7.8), and the suspensions were then forced twice through a French pressure cell (American Instrument Co., Silver Spring, Md.) operated at 100 MPa. Extracts were microcentrifuged, and the supernatants were collected. All these operations were performed at 0 to 4°C. CAT assays were performed as previously described by Shaw (47). The quantity of protein was determined by the method of Bradford (5) with a Bio-Rad dye reagent, using ovalbumin as the standard.

For β-galactosidase assays, cells were collected by centrifugation and resuspended in ice-cold water. Accurate measurements of the turbidity of the cell suspensions were made. The β-galactosidase activity was measured in sodium dodecyl sulfate-chloroform-permeabilized cells; the enzyme assay and units used are those described by Miller (39). Each measurement of CAT or β-galactosidase activity was performed three times. The standard deviations of the mean values were within ±15%.

RESULTS

Localization of the *bet* promoters. For localization of the *bet* promoters, various fragments of the *bet* region were subcloned into the multicopy vector pKK232-8 (6) in order to generate operon fusions with its promoterless *cat* gene. The extent and orientation of the *bet* fragments tested are shown in Fig. 1. Fusions with *cat* were made within the *betT*, *betI*, and *betA* genes. The fusion plasmids pTB2215, pAB4754, and pAB4764 carried the full-length *betI*, but in plasmid pAB4764, *betI* was disrupted by an insertion of a *Bam*HI linker into its *Cla*I site. The other fusion plasmids listed carried either only small or no fragments of *betI*.

All plasmids were tested in stressed cells of the *bet* deletion mutant FF2005 grown in medium 63 containing 0.3 M NaCl. In order to simultaneously examine the influence of *betI*$^+$ in *trans* (see below), the CAT activities were measured with cells which in addition to the *cat* fusion plasmids also carried either plasmid pTP100 (*betI*$^+$) or plasmid pTP101 (*betI* negative control) derived from pJRD184 (20). CAT-fusion plasmids carrying a functional *betI*$^+$ in *cis* were tested only together with pTP101 (Fig. 1; BetI regulation).

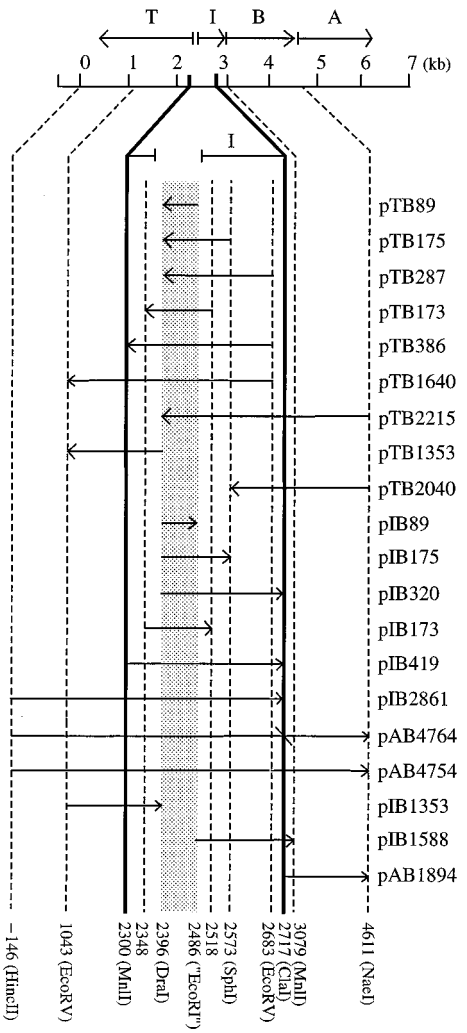
The smallest *bet* fragment tested which conferred CAT activity was an 89-bp fragment extending from a *Dra*I site to a genetically engineered *Eco*RI site (Fig. 1). These sites were 28 and 11 nucleotides upstream of the deduced translational startpoints of *betT* and *betI*, respectively (29) (see Fig. 2B). The 89-bp fragment displayed promoter activity in both the *betT* (plasmid pTB89) and *betI* (plasmid pIB89) directions. All fragments shown in Fig. 1 which encompassed this region conferred promoter activity, whereas the fragments lacking this region did not. Measured as the CAT activity in the absence of BetI, there was no major difference in the basis promoter strength between the *betT* and *betI* fusions. Furthermore, the CAT activity produced was not much influenced by the length of the *bet* sequences extending outside the 89-bp region.

BetI repression and BetI operator region. Compared with the cells carrying pTP101, the presence of pTP100 (*betI*$^+$) in the cells resulted in a strong reduction of the CAT activities expressed from the promoter-active fusion plasmids (Fig. 1; BetI regulation). For plasmids with the 89-bp fragment (see above), the reduction was sevenfold for the fusion with *betT* (pTB89) and eightfold for the fusion with *betI* (pIB89). In general, the observed repression of CAT activity was even more pronounced for fusion plasmids with larger promoter-active fragments, e.g., plasmids pTB173 and pIB173 with a 173-bp fragment displayed more than a 20-fold reduction. These differences in BetI-dependent repression of large and small promoter fragments were observed consistently and may be due to the nature of BetI binding, as discussed in the accompanying paper (44).

Similarly, promoter-active fusion plasmids with a *betI*$^+$ gene in its native *cis* configuration conferred less CAT activity than plasmids with a comparable promoter fragment but lacking *betI*$^+$. This is evident by comparing CAT activities conferred by the *betA* fusions of plasmids pAB4754 (*betI*$^+$) and pAB4764 (*betI*) and by comparing the CAT activities conferred by the *betT* fusions of plasmids pTB2215 (*betI*$^+$) and pTB287 (*betI*) (Fig. 1). Thus, BetI repressed the *betT* and *betI* promoters when present both in *cis* and in *trans*, and the main operator appeared to be within the 89-bp fragment.

Osmotic induction and choline regulation. Cells of FF2005 carrying a *cat* fusion plasmid together with plasmid pTP100 (*betI*$^+$) or pTP101 (control) were grown in LOM and then exposed for 3 h to 0.3 M NaCl alone or 0.3 M NaCl together with 1 mM choline. The CAT activities displayed by cells carrying plasmid pIB89 (*betI*::*cat*) or pTB89 (*betT*::*cat*) are listed in Table 2. Cells carrying pTP100 (*betI*$^+$) displayed reduced CAT activities under all growth conditions tested compared with cells with the control plasmid pTP101. For cells carrying pTP101 (control), the osmotic induction was threefold but was slightly reduced by the addition of choline. In comparison, the CAT activities observed with cells carrying pTP100 (*betI*$^+$) were increased twofold by osmotic stress alone and sevenfold by the combination of osmotic stress and choline.

Experiments performed with cells carrying fusion plasmids with longer promoter-active *bet* fragments displayed a similar pattern of regulation. Thus, these data indicated that the choline regulation, but not the osmotic regulation, of the *bet* promoters depended on BetI. To rule out the possibility that the osmotic induction observed was a general copy number effect caused by the vector pKK232-8, a *tac* promoter was cloned in front of the *cat* gene in pKK232-8. Cells of FF2005 carrying



	BetI regulation			ArcA regulation		
	betI	*betI*$^+$	Ratio	*arcA*	*arcA*$^+$	Ratio
pTB89	4.4	0.62	7	19	6.4	3
pTB175	4.8	0.36	13	−	−	
pTB287	5.5	0.29	19	22	7.3	3
pTB173	3.5	0.17	21	12	0.32	39
pTB386	3.2	0.13	25	16	0.36	46
pTB1640	6.7	0.16	42	−	−	
pTB2215	−	0.37		−	−	
pTB1353	0	0		−	−	
pTB2040	0	0		−	−	
pIB89	5.2	0.62	8	10	4.7	2
pIB175	14.8	1.7	9	18	4.0	5
pIB320	6.3	0.71	9	12	4.0	3
pIB173	6.1	0.28	22	17	0.64	27
pIB419	5.3	0.26	20	22	0.64	32
pIB2861	8.3	0.58	14	14	0.68	21
pAB4764	7.3	0.23	32	−	−	
pAB4754	−	0.94		−	−	
pIB1353	0	0		−	−	
pIB1588	0	0		−	−	
pAB1894	0	0		−	−	

FIG. 1. Localization of *bet* promoters and influence of BetI and ArcA on *bet* promoter activities. In the schematic presentation on the left, the top arrows show the organization of the *bet* genes. The arrows below show the extension and orientation of *bet* fragments linked to a *cat* reporter gene on plasmid pKK232-8. The coordinates at the bottom correspond to the numbering of nucleotides published previously by Lamark et al. (29). For *Mnl*I, the coordinates given correspond to its cleavage site, whereas for the other restriction enzymes the coordinates given correspond to the first nucleotide of the recognition sequence. *Eco*RI is an artificial *Eco*RI site which was generated in vitro and used for construction of plasmids pTB89 and pIB89. Plasmid pAB4764 carried a *Bam*HI linker in the *Cla*I site of *betI*. The table on the right shows the names of the *cat* fusion plasmids and the CAT activities which they expressed. Data for BetI regulation were obtained with FF2005 carrying a *cat* fusion plasmid together with pTP100 (*betI*$^+$) or pTP101 (*betI*). The cells were grown aerobically in medium 63 with 0.3 M NaCl added. Data for ArcA regulation were obtained with FF2005 (*arcA*$^+$) or TT50 (*arcA*) carrying the *cat* fusion plasmids. The cells were grown anaerobically in medium 63. CAT activity units are nanomoles per minute per milligram of cell protein. Each value represents the average of three independent experiments. −, not measured.

this plasmid did not display any osmotic induction of the CAT activity (data not presented).

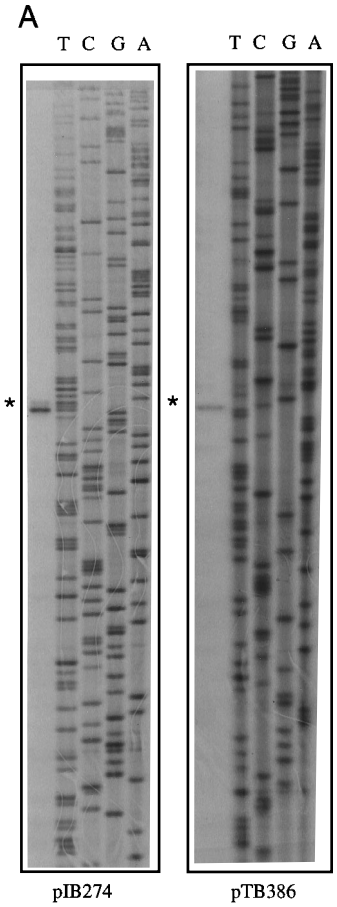
Primer extension analysis. In order to identify the 5′ end of the *betIBA* and *betT* transcripts, a primer extension analysis was conducted with RNA isolated from FF2005 carrying various fusion plasmids. The plasmids used (see below) are depicted in Fig. 1, except pIB274, which is a *betI*::*cat* fusion plasmid carrying *bet* coordinates 2300 to 2573. The cells were grown in medium 63–0.3 M NaCl prior to RNA extraction, and two different primers with their 3′ ends within the polylinker or within the *cat* gene of the vector pKK232-8 were used to determine the transcriptional start points.

The primer extension data obtained with RNA from FF2005 (pTB386) (Fig. 2A) and FF2005(pTB89) were the same with both primers tested. The only major extension product seen, which was also the largest product visible, corresponded to an initiation of the *betT* transcript at an A residue 42 bp upstream

TABLE 2. Osmotic regulation and BetI-dependent choline induction of the *betI* and *betT* promoters residing on an 89-bp fragment linked to the *cat* gene of vector pKK232-8[a]

Plasmids[b]	CAT activities[c] when cells grown in LOM:		
	Alone	+ 0.3 M NaCl	+ 0.3 M NaCl + 1 mM choline
pIB89 (*betI*::*cat*) and pTP100 (*betI*$^+$)	0.20	0.40	1.1
pIB89 (*betI*::*cat*) and pTP101 (control)	0.62	2.1	1.8
pTB89 (*betT*::*cat*) and pTP100 (*betI*$^+$)	0.17	0.33	1.1
pTB89 (*betT*::*cat*) and pTP101 (control)	0.71	2.4	1.9

[a] The host strain was FF2005 (Δ*bet*).
[b] BetI was supplied in *trans* from pTP100 (*betI*$^+$). pTP100 (*betI*$^+$) and pTP101 (control) were derived from pJRD184 (20).
[c] CAT activities are expressed in nanomoles per minute per milligram. The values are the averages of three independent experiments.



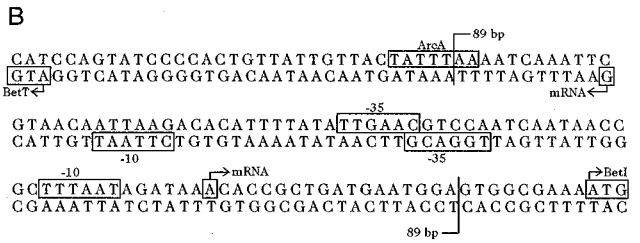


FIG. 2. An example of primer extension analysis of *betI* and *betT* transcripts. (A) 5′ ends of *betI* (left panel) and *betT* (right panel) mRNA were mapped by primer extension as described in Materials and Methods. *betI* and *betT* mRNAs were obtained from FF2005(pIB274) and FF2005(pTB386), respectively. *, location of the main primer extension product. (B) DNA sequence of the promoter region. The transcriptional start points indicated by the primer extension analysis, the most probable −35 and −10 boxes, the deduced translational start codons of BetI and BetT, and a putative ArcA binding site are boxed. The borders of the 89-bp promoter-active region, found in plasmids pIB89 and pTB89, are indicated by brackets.

of the deduced start codon of *betT*. Experiments with FF2005 (pIB274) (Fig. 2A) also yielded a single prominent extension product, corresponding to an initiation of the *betIBA* transcript at a G residue 27 bp upstream of the deduced start codon of *betI*. In addition, a minor extension product which was two residues longer than the major *betIBA* product was found. This extra band was observed with both primers tested. In experiments with FF2005(pIB2861), which consistently yielded smaller amounts of extension products than FF2005(pIB274), only the stronger band was seen. The presence of BetI, supplied from plasmid pTP100, did not change the transcriptional start points from plasmid pIB274 (data not shown).

The deduced start points for *betI* and *betT* transcription were located within the 89-bp region (Fig. 2B). Assuming that the mRNA used in our primer extension analysis was not processed, the most likely −35 and −10 boxes of the *betI* promoter were TTGAAC(17)TTTAAT, which are similar to those previously suggested from the DNA sequence (29). The most probable −35 and −10 boxes for the *betT* promoter were TGGACG(17)CTTAAT (Fig. 2B).

Construction of a *betI* mutation on F′2. *lacZ* operon fusions have previously been generated in the three structural *bet* genes which reside on plasmid F′2 (16). However, all these fusion mutants carry $betI^+$ in its native *cis* configuration. In order to mutate *betI* on F′2, we constructed plasmid pTL300. The 5.5-kb insert of this plasmid carried the *bet* promoter region and the *betI*::*cat* fusion of plasmid pIB2861 (Fig. 1), a Tc^r marker linked to the 3′ side of the *cat* gene for selection purposes, and a chromosomal DNA fragment of 1.3 kb which originated from the downstream region of *betA*. This insert was transformed into strain TT42 [F′2 (*betT-lacZ* $betIBA^+$ Kn^r) Δ*bet recD*], and a *betI*::*cat* fusion on F′2 was obtained by homologous recombination, as illustrated in Fig. 3. The structure of the mutant F′2 (*betT-lacZ betI*::*cat* Δ*betBA* Kn^r Tc^r) of TT44 was verified by Southern blot analysis (data not shown).

Expression of *bet* genes residing on plasmid F′2. Strain FF914, which carried plasmid F′2 ($betIBA^+$ *betT-lacZ*) generated by Eshoo (16), displayed a low background activity of β-galactosidase when grown in LOM. The β-galactosidase activity was increased 2.5- and 7-fold, respectively, when the cells were exposed for 3 h to LOM with 0.3 M NaCl added or to LOM with 0.3 M NaCl and 1 mM choline added (Table 3). The addition of NaCl to concentrations of NaCl above 0.3 M did not increase the osmotic induction of *betT* expression, and medium 63 without NaCl added caused a partial induction (data not shown). This mode of induction of *betT* was in good agreement with data published previously by Eshoo (16).

When grown in LOM, strain TT44, which carried plasmid F′2 (*betI*::*cat betT-lacZ*), displayed a twofold-higher background activity of β-galactosidase than strain FF914 ($betI^+$ *betT-lacZ*). The β-galactosidase activity produced by TT44 was increased fivefold when the cells were exposed to osmotic stress (0.3 M NaCl), but the activity was not further increased by the addition of choline (Table 3). The measurements of enzyme activities expressed from the *betT-lacZ* and *betI*::*cat* fusions of TT44 (Table 3) confirmed the data obtained with

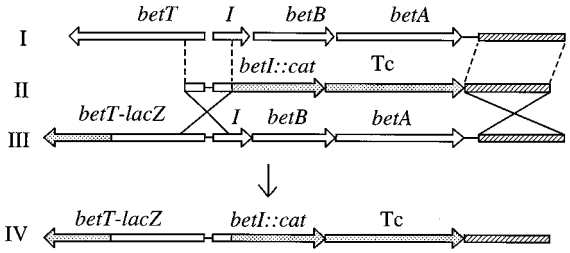


FIG. 3. Insertion of *betI*::*cat* operon fusion by linear transformation. The schematic presentation shows the organization of the *bet* genes (I); the *bet* fragments, *cat* reporter gene, and Tc^r marker (Tc) of the 5.5-kb linear fragment (from pTL300) used in transformation (II); the *bet* genes and *lacZ* reporter gene of recipient F′2 (*betT-lacZ*) (III); and the *bet* region of recombinant F′2 (*betT-lacZ betI*::*cat*) formed by a double crossover (IV). *I*, *betI*. Symbols: open bars and arrows, *bet* genes; hatched bars, downstream region of *betA*; stippled arrows, resistance or reporter genes (*cat*, Tc, and *lacZ*; not drawn to scale). The Kn^r marker (see the text) is located downstream to the left of *lacZ* and is not shown in the figure.

TABLE 3. Osmotic induction and choline induction of *bet* operon fusions residing on plasmid F′2[a]

Strain	Genotype of F′2	β-Galactosidase and CAT activities[b] when cells grown in LOM:		
		Alone	+ 0.3 M NaCl	+ 0.3 M NaCl + 1 mM choline
TT44	Δ*betBA betT-lacZ betI*::*cat*	530 (70)	2,400 (300)	2,300 (300)
FF914	*betIBA*$^+$ *betT-lacZ*	290	740	1,900
TT44(pTP200)[c]	Δ*betBA betT-lacZ betI*::*cat*	60 (6)	160 (4)	390 (30)
FF914(pTB89)[d]	*betIBA*$^+$ *betT-lacZ*	510	2,000	1,900
TT64 (*hns*)	Δ*betBA betT-lacZ betI*::*cat*	610 (60)	1,600 (210)	1,600 (190)
TT69 (*hns rpoS*)	Δ*betBA betT-lacZ betI*::*cat*	440 (80)	2,500 (380)	— (—)

[a] Relevant descriptions for strains and genotypes of F′2 are given.
[b] β-Galactosidase activities (in Miller units) are listed outside parentheses. CAT activities (in piconomoles per minute per milligram) are listed in parentheses. The values are the averages of three independent experiments. —, not measured.
[c] Plasmid pTP200 carried *betI*$^+$.
[d] Plasmid pTB89 carried the *bet* promoter and operator regions.

multicopy-plasmid-carrying strains (Table 2), showing that in cells lacking BetI, expression of the *bet* promoters was stimulated by osmotic stress but not by choline.

The multicopy plasmid pTP200 was used to supply *betI*$^+$ in *trans*. The expression of both *betI*::*cat* and *betT-lacZ* of strain TT44(pTP200) was strongly reduced under all growth conditions tested compared with that for strain TT44 without pTP200. However, the β-galactosidase activity displayed was osmotically inducible and was further induced by osmotic stress plus choline. The CAT activities of TT44(pTP200) were at the background level, but cells grown with osmotic stress plus choline displayed some activity (Table 3). It should be noted that in plasmid pTP200, an RNA terminator was inserted upstream of the *betI*$^+$ fragment; thus, *betI*$^+$ was presumably expressed only from its native promoter. pTP200 caused less repression than plasmid pTP100 (used in experiments described above), which lacked a terminator in front of *betI*$^+$ (data not presented).

Plasmid pTB89 carried the 89-bp promoter fragment with the operator for BetI binding (see above) (44). Cells of FF914(pTB89) containing F′2 (*betIBA*$^+$ *betT-lacZ*) displayed a level of production of β-galactosidase which was different from that of FF914 without pTB89; i.e., the background activity in LOM was increased and the osmotic induction remained, but the choline induction was absent. This mode of expression was the same as for TT44 (*betI*) (Table 3). Apparently, the presence of multiple copies of the BetI operator region titrated out BetI produced in FF914(pTB89). If there exists a transacting factor for osmoregulation which binds within the 89-bp fragment, it was evidently not titrated out in the present experiment.

Effects of *hns* (*osmZ*) mutation and carbon source. It has been reported previously that strains which carry an *hns* null mutation grow slowly and that a second null mutation in *rpoS* partially suppresses this phenotype (3). The data presented in Table 3 show that both *bet* promoters remained osmotically regulated when expressed from plasmid F′2 (*betI*::*cat betT-lacZ*) in an *hns* (strain TT64) or an *hns rpoS* (strain TT69) background. For TT69, the observed promoter activities were the same as for strain TT44 (*hns*$^+$ *rpoS*$^+$). Thus, the somewhat reduced *bet* expression observed for TT64 at high osmolarity was probably only an indirect effect of the *hns* mutation. It has been shown previously that *rpoS* does not influence *bet* expression (25).

When FF914 was grown in LOM with maltose (5 mM) or glycerol (20 mM) as the carbon source, the expression of *betT-lacZ* was similar to that found with glucose (data not shown). Thus, in the absence of osmotic stress, the reduced growth rate caused by poorer substrates did not cause induction of *betT*.

Oxygen regulation by ArcA. We tested the influence of *arcA* and *fnr* on the production of β-galactosidase from plasmids F′2 (*betIBA*$^+$ *betT-lacZ*) and F′2 (*betIB*$^+$ *betA-lacZ*). All strains were grown under aerobic and anaerobic conditions in medium 63. The cells were not subjected to osmotic stress, since osmotically stressed cells, particularly those containing *arcA*, grew poorly under anaerobic conditions.

Compared with aerobic growth conditions, anaerobic growth of *fnr*$^+$ *arcA*$^+$ control cells caused five- and sevenfold repression of *betA-lacZ* (strain FF33) and *betT-lacZ* (strain FF914) expression, respectively (Table 4). This is consistent with previous findings for *bet* fusions (16). Under aerobic growth conditions, the expression of the *bet-lacZ* fusions was not influenced by the presence of an *fnr* or an *arcA* mutation. But in anaerobically grown cells, *fnr* caused a partial derepression of the *betA* and *betT* genes, and *arcA* caused a complete derepression of these genes (Table 4). Such a pattern for derepression by *fnr* and *arcA* has been reported previously for other genes (*sodA* and *arcA*) which are directly regulated by ArcA and only indirectly regulated by Fnr (10).

In order to identify the parts of the *bet* region that are involved in this ArcA-mediated oxygen regulation, the CAT activities which were produced from several of our multicopy fusion plasmids in cells of FF2005 (*recA arcA*$^+$) and TT50 (*recA*$^+$ *arcA*::Tn*10*) were compared. These strains were isogenic except for the mutations mentioned. (The *recA* mutation does not influence *bet* expression.) Cells were grown in medium 63 under anaerobic conditions. It should be noted that the cells used in these experiments did not carry plasmids pTP100 (*betI*$^+$) or pTP101 and were grown under different conditions than those used in the experiments described above.

TABLE 4. Regulation of *bet* operon fusions residing on F′2 by oxygen, ArcA, and Fnr[a]

Strain	Genotype of F′2	β-Galactosidase activity[b]		Ratio[c]
		Aerobic growth	Anaerobic growth	
FF33	*betA-lacZ*	180	35	5
TT54 (Δ*fnr*)	*betA-lacZ*	200	85	2
TT52 (*arcA*::Tc^r)	*betA-lacZ*	220	200	1
FF914	*betT-lacZ*	400	55	7
TT53 (Δ*fnr*)	*betT-lacZ*	410	120	3
TT51 (*arcA*::Tc^r)	*betT-lacZ*	390	380	1

[a] Relevant descriptions for strains and genotypes of F′2 are given.
[b] β-Galactosidase activities are given in Miller units. The cells were grown in medium 63, and the values are the averages of three independent experiments.
[c] Ratio of β-galactosidase activity under aerobic growth to that under anaerobic growth.

Thus, the data on ArcA regulation, presented in Fig. 1, are not directly comparable to the data on BetI regulation presented in the same figure.

Some of the plasmids tested (i.e., pIB173, pIB419, pIB2861, pTB173, and pTB386) conferred 21- to 46-fold-higher CAT activity in the *arcA* background than in the *arcA*$^+$ background, and expression from these plasmids was strongly repressed in anaerobic *arcA*$^+$ cells. Expression from the other plasmids tested (i.e., pIB89, pIB175, pIB320, pTB89, and pTB287) conferred only two- to fivefold-higher activity in the *arcA* background, and these plasmids conferred a high level of activity also in the *arcA*$^+$ cells. Thus, full ArcA regulation was not seen with the plasmids carrying the 89-bp promoter fragment (pIB89 and pTB89), but it was seen with the 173-bp fragment, both in the *betT* (pTB173) and *betI* (pIB173) directions. Upon inspection of the data presented in Fig. 1, it is evident that all fully ArcA-regulated plasmids carried a 51-bp region, which is not part of the 89-bp region and is situated on the *betT* side of the 173-bp fragment.

DISCUSSION

The function of the choline-to-betaine pathway of *E. coli* is to produce the osmoprotectant betaine. Neither the product nor the precursors are catabolized by the cells. Due to the O_2 requirement of choline dehydrogenase, *E. coli* can utilize choline only under aerobic growth conditions (31). Thus, there are obvious reasons for the organism to regulate the pathway in response to oxygen, choline, and hyperosmotic stress. In the present investigation, we have dissected the *bet* promoters and shown that these three stimuli regulate the gene expression by three separate mechanisms. Oxygen and choline exert their control via the transacting DNA-binding proteins ArcA and BetI, respectively. ArcA is known to control the expression of a number of oxygen-inducible genes of *E. coli* (24), whereas BetI is a specific choline-sensing repressor for the *bet* regulon. No regulatory protein which is required for the osmotic induction of *bet* has been identified, and the osmotic signal for *bet* induction remains unknown.

The present cloning analysis with *cat* operon fusions on multicopy plasmids showed that a DNA fragment of 89 bp contains the divergent *betT* and *betI* promoters as well as the main operator site for BetI. Because of the rather unusual organization of the *betIBA* operon, with the regulatory gene situated in front of the structural genes, a search was made for internal promoters. No additional promoter region was found outside of the 89-bp fragment. The present primer extension analysis indicated that the transcriptional start points of the divergently organized *betT* and *betI* genes were 61 nucleotides apart. The entire promoter regions of *betI* and *betT* showed a rather high degree of homology, with 20 of 40 residues upstream of the transcriptional start points being identical. The putative −35 boxes of the promoters were partially overlapping and covered regions of dyad symmetry. In the accompanying paper, we report that this is the binding site for BetI (44).

The notion that the osmotic induction of both *betI* and *betT* does not require BetI was unambiguously proven in experiments with reporter genes on plasmid F′2. In fact, in the absence of choline, the osmotic induction of *betT* was lower in *betI*$^+$ cells than in *betI* cells. Apparently, *betI*$^+$ caused repression of the *bet* promoters at both high and low osmolarity, but more so at high osmolarity when BetI production is higher. Results for osmotic gene regulation obtained with multicopy plasmids should be interpreted with some caution. It is, however, remarkable that the 89-bp promoter fragment, as well as all the larger promoter-containing fragments tested, displayed osmotic induction of the *betI* and *betT* promoters in the absence of BetI, which was in accordance with observations made with F′2.

On the basis of the finding that the *bet* promoters residing on F′2 remained fully osmotically regulated in an *hns*::Ap^r *rpoS*::Tn*10* background, we conclude that *hns* does not influence *bet* expression directly. Mutations in *hns* were previously shown to affect the expression of a large number of genes, including several which are osmotically inducible (3, 18, 21, 53). The *proU* system has often been used in studies of osmotic induction of gene expression, particularly because this system displays a much higher degree of induction than other osmoregulated genes. A negative regulatory element (NRE) is located within the first gene of the *proU* operon and constitutes a specific binding site for H-NS (13, 34, 41, 42). With NRE deleted, the basal *proU* expression at low osmolarity increases, and the remaining level of the osmotic induction is about 10-fold (13, 38, 41, 42). We did not find any evidence in our cloning and expression analysis that the *bet* region contained any *cis* transcriptional elements other than those for BetI and ArcA binding. A presumed pleiotropic effect of *hns* was a reduced *bet* expression at high osmolarity, similar to that which was observed with the *proU* promoter from which NRE had been deleted located on the chromosome (38).

The deduced −10 and −35 boxes of the *bet* promoters do not resemble those of the *proU* promoter (17, 48). However, the *betT* promoter displays similarity in the region upstream of the −10 box, including a TG motif at −14 and −15. This TG motif is important for *proU* expression (38) and was first recognized for the extended −10 region of altered λp_{RE} and *galP1* promoters (26, 28). The *betI* promoter displays less similarity to the *proU* promoter, but it contains the −14 G. The observed degree of osmotic induction of the *bet* promoters (about fivefold) when they reside on plasmid F′2 in a *betI* background was not far from that found for the *proU* promoter with a deletion of NRE. For the *proU* promoter with a deletion of NRE, the remaining induction seems to require the HU nucleoid protein, although mutations in *hup* genes encoding HU subunits are highly pleiotropic (36). Alternatively, it has been proposed that the ionic changes in stressed cells may contribute to an induced expression from promoters which are resistant to high cytoplasmic ionic concentrations (38).

In mammals, the synthesis of betaine from choline occurs in the mitochondria (27). Therefore, the finding that the *bet* regulon belongs to the ArcA modulon is in accordance with a well-known pattern which has been pointed out previously, namely, that this two-component regulatory system often controls *E. coli* functions which have their counterparts in the mammalian mitochondria (23). The present analysis showed that ArcA repressed *bet* promoters which were situated on both single and multicopy plasmids. The main binding site(s) for ArcA appeared to be at the *betT* side of the promoter region, either within a 51-bp region which is adjacent to the 89-bp promoter region or, more likely, at the *Dra*I site separating these regions. On the *betI* strand, this *Dra*I site is part of a TATTTAA sequence (Fig. 2B), which recently has been suggested as the consensus for ArcA binding (15). In vitro studies performed with other ArcA-regulated gene systems show that ArcA may bind to more than one site on DNA (15, 52). Several putative ArcA binding sites with one or two mismatches are located within the 89-bp *bet* region, which may account for the weak ArcA regulation observed for this fragment. The *bet* promoter sequence does not contain any consensus sequence for Fnr binding.

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