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 osmoequilibrium 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. Although 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 global 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^5 factor (RpoS), which is induced by osmotic stress at the level of translation (3, 33). The basal level of σ^5 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^5 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).
**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 into 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 A₆₀₀ of 0.5 to 1.0. Anaerobic cultures were grown to an A₆₀₀ 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 (arg-F-lac)₁₆₉₅ insertion 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 hnsΔ(T₃) (ampicillin resistance) were always performed with newly constructed strains. Strain GM161 was used for isolation of plasmids without Dam methylation.

Selecting for Kan² (kanamycin resistance) and Ap², the plasmids F₂ (Kan²) of MLE33 and MLE914 carrying bet-locl fusions (16) were conjugated into strain DH5F⁻ (Sm²) carrying pGEM-3Zf(+) (Ap²; Promega). From this strain and selecting for Kan² and Sm² (streptomycin resistance), the plasmids F₂ were further conjugated into MC4100, creating FF33 and FF914, and into FC2, creating TT54 and TT53. For construction of strain TT42, (arg-F-lac)₁₆₉₅ 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 hnsΔ(T₃) (ampicillin resistance) were always performed with newly constructed strains. Strain GM161 was used for isolation of plasmids without Dam methylation.

**Plasmid constructions.** Several DNA fragments from the bet region were inserted into the polynucleotide of vector pKK223-8 (6). Except for the fragment of plasmid pBB74 (described in Results), the extent and orientation of all these bet fragments are shown in Fig. 1. The 173-bp bet fragment of plasmids pBI173 and pBB173 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 pAB7464 was made by insertion of a BamHI linker (GGGATCC) (Boehringer Mannheim) into the ClaI site obtained from the gene (pAB7463). The resulting defected bet gene.

An E. coli BglII fragment (coordinates 2049 to 4257) was cloned into M13, and an artificial E. coli site was created 11 bp upstream of the Bet coding region by in vitro mutagenesis by the method of Su and GeWey (51). The mutagenesis changed the original sequence (coordinates 2484 to 2493) from TGGATG GCGC to TGGATCCG (the new EcoRI site is underlined). The resulting mutagenesis was verified by DNA sequencing. The new EcoRI site was used to construct plasmids pIB89 and pIB89 (see Fig. 1) and plasmid pFF440, which was used in the studies described in the accompanying paper (44). pFF440 contains a 1.6-kb EcoRI-XhoI fragment (encoding bet coordinates 2486 to 4257) cloned into vector pGEM-7Zf(–).

Plasmid pTP100 was made by inserting an E. coli BglII fragment (coordinates 2049 to 4257) from plasmid pFP221 (1) into the E. coli-HincII sites of plasmid pJR184 (20). Control plasmid pTP101 was made by insertion of a 0.3-kb HincII-EcoRI fragment from the polynucleotide of pUC119 (Pharmacia) into the corresponding sites of pJR184. For construction of plasmid pTP200, the E. coli-BglII fragment of pFP221 was cloned into the E. coli-BamHI sites of pJR184; this was followed by an insertion of the transcriptional terminator T1 from pKK232-8 (180-bp EcoRI fragment) into the E. coli site of the resulting plasmid.

**Insertion of bet-locl on plasmid F₂.** Plasmid pTL300 was constructed in order to insert a bet-locl fusion into the bet genes on F₂. A 1.8-kb KpnI-BamHI fragment containing the 3′ end of bet₄ and its downstream region was isolated from plasmid pFP221 and digested into the polynucleotide of pGEM-7Zf(–). A 1.3-kb PstI fragment containing the 3′ end of the cat gene was purified from pKK232-8 and ligated into the EcoRV site of the chromosomal fragment in the same orientation as the bet₄ gene. The EcoRV site was located downstream of bet₄ and 1.3-kb from BamHI. The resulting plasmid was linearized with EcoRI, which cut in the polynucleotide of the vector and in the cat gene, and a 0.9-kb EcoRI fragment containing the 5′ end of the bet-locl fusion of pLB2001 (see Fig. 1) was inserted. This created a plasmid insert containing a complete bet-locl fusion.
starting with a bet fragment of 625 bp and ending with a 1.3-kb EcoRV-BamHI fragment from the chromosomal region downstream of betA. We have found by DNA sequencing that this 1.3-kb fragment contains a Dam-methylated ClaI site 3 bp downstream of the EcoRV site. To make plasmid pTL300, a 2.1-kb EcoRI-PvuII fragment containing the Tc' marker of pBR322 was cloned into this ClaI site.

The 5.5-kb insert of pTL300 (see Fig. 3) was excised with XhoI and NotI and inserted into plasmid F2 (bet-lacZ) of TT42 (ΔbetRD) by transformation, as described previously for recD-containing strains (45). F2 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 restriction markers of the 5.5-kb fragment are integrated in the bet region on F2, will have a Bet' phenotype because of a deletion of the betA genes, as illustrated in Fig. 3. Therefore, we selected for Tc' transformants and scored for a Cm' (chloramphenicol resistance) Kn' 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 F2 (betI[A' bet'-]), to Kn'. Of 30 Kan' transductants tested, 24 carried the three markers Tc-, Cm', and Bet- (e.g., TT44; Table 1), whereas the remaining six were Tc' Cm' Bet'. These data showed that Kan' (betI-lacZ), betI-cat, and Tc' are linked on F2'. As would be expected for a construct in which the cat gene is under the control of the betI promoter, the Cm' conferred by strains carrying this fusion was rather weak. Therefore, to prevent selection of betI promoter mutations which increased the CAT activity, Cm' was never used as a selection marker.

To verify the structure of the mutant F2', whole-cell DNA was isolated from strains TT44 (mutant F2') and TT42 (negative control) and digested with BamHI, BamHI-EcoRI, or EcoRV. Southern blot analysis was performed according to the methods described by Ausubel et al. (2), and the hybridizations were done 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), using radiolabelled [γ-32P]dATP (Amersham) and T4 polynucleotide kinase (Amersham). The DNA primers were used: CAAGGCCTCCTCGATGTTCTC TACTCTGGCAATGGATATTAACGGTGG, which had 9 bp ends within the polylinker and the cat gene, and which, respectively, of vector pKK232-8. Radiolabelled probes 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. The reduction was determined using ovalbumin as the standard.

**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 BamHI linker into its ClaI site. The other fusion plasmids listed carried either small or no fragments of bet.

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 pRJD184 (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 DraI site to a genetically engineered EcoRI site (Fig. 1). These sites were 28 and 11 nucleotides upstream of the deduced translational start-points 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 pBB89) 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 (pBB89). 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 pBB173 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 bet A 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 pBB89 (betI'cat) or pBB89 (betIcat) 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 betI 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 lac promoter was cloned in front of the cat gene in pKK232-8. Cells of FF2005 carrying
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 of the polylinker, which is consistent with the sequencing data obtained at the 5’ end of the betT transcript. The primer extension data obtained with RNA from FF2005 (pTB1640) 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 of the polylinker, which is consistent with the sequencing data obtained at the 5’ end of the betT transcript.

![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 the first nucleotide of the recognition sequence. EcoRI is an artificial EcoRI site which was generated in vitro and used for construction of plasmids pTB89 and pIB89. Plasmid pAB4764 carried a BamHI linker in the ClaI site of bet. 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 betI 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.](http://jb.asm.org/)
the borders of the 89-bp promoter-active region, found in plasmids pIB89 and pTB89, are indicated by brackets.

transformed into strain TT42 [F'2 (betT-lacZ betIBA + K n') Δbet 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 betIBA K n' Tc') 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

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 TGGGAC(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 betT' 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 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
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 bet\textsuperscript{I} in trans. The expression of both bet::cat and bet::T-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 bet\textsuperscript{I} fragment; thus, bet\textsuperscript{I} 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 bet\textsuperscript{I} (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 (betIB\textsuperscript{A}’ 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\textsuperscript{−} rpoS\textsuperscript{−}). 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 strain 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 (betIB\textsuperscript{A}’ betT-lacZ) and F’ 2 (betIB\textsuperscript{A}’ 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\textsuperscript{−} arcA\textsuperscript{+} 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\textsuperscript{−}) and TT50 (recA\textsuperscript{−} arcA::Tn10) 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 (bet\textsuperscript{I}’) or pTP101 and were grown under different conditions than those used in the experiments described above.

**TABLE 3. Osmotic induction and choline induction of bet operon fusions residing on plasmid F’ 2**

| Strain     | Genotype of F’ 2 | β-Galactosidase and CAT activities\(^a\) when cells grown in LOM: |
|------------|------------------|-----------------------------------------------------------------|
|            |                   | Alone   | + 0.3 M NaCl | + 0.3 M NaCl + 1 mM choline |
| TT44       | ΔbetBA betT-lacZ betI::cat | 530 (70) | 2,400 (300) | 2,300 (300) |
| FF914      | betIB\textsuperscript{A}’ betT-lacZ | 290 | 740 | 1,900 |
| TT44(pTP200)\(^d\) | ΔbetBA betT-lacZ betI::cat | 60 (6) | 160 (4) | 390 (30) |
| FF914(pTB89)\(^d\) | betIB\textsuperscript{A}’ betT-lacZ | 510 | 2,000 | 1,900 |
| TT64 (hns) | ΔbetBA betT-lacZ betI::cat | 610 (60) | 1,600 (210) | 1,600 (190) |
| TT9 (hns rpoS) | ΔbetBA betT-lacZ betI::cat | 440 (80) | 2,500 (380) | — (—) |

\(^a\) Relevant descriptions for strains and genotypes of F’ 2 are given.

\(^d\) β-Galactosidase activities (in Miller units) are listed outside parentheses. CAT activities (in picomoles per minute per milligram) are listed in parentheses. The values are the averages of three independent experiments. —, not measured.

Plasmid pTP200 carried bet\textsuperscript{I}.

Plasmid pTB89 carried the bet promoter and operator regions.

**TABLE 4. Regulation of bet operon fusions residing on F’ 2 by oxygen, ArcA, and Fnr\(^b\)**

| Strain     | Genotype of F’ 2 | β-Galactosidase activity\(^a\) |
|------------|------------------|-------------------------------|
|            |                  | Aerobic growth | Anaerobic growth |
| FF933      | betA-lacZ        | 180 | 35 | 5 |
| TT54 (Δfnr) | betA-lacZ        | 200 | 85 | 2 |
| TT52 (arcA::Te\textsuperscript{c}) | betA-lacZ | 220 | 200 | 1 |
| FF914      | betT-lacZ        | 400 | 55 | 7 |
| TT53 (Δfnr) | betT-lacZ        | 410 | 120 | 3 |
| TT51 (arcA::Te\textsuperscript{c}) | 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. 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 osmoregulation of betaine. Neither the product nor the precursors are catabolized by the cells. Due to the O2 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 betT and betI 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 betT cells than in betI cells. Apparently, betT caused repression of the bet promoters at both high and low osmolality, but more so at high osmolality 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 longer 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* proS::Tn10* 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 osmo-regulated 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 osmolality 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 osmolality, 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 *apet* 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 betI promoters (about five-fold) 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 *hns* nucleoid protein, although mutations in *hup* genes encoding HU subunits are highly pleiotropic (36). Alternatively, it has been proposed that the ion 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 betI 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 DraI site separating these regions. On the betT strand, this DraI site is part of a TATAATA 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.
