JOURNAL OF BACTERIOLOGY, Nov. 1988, p. 5208–5215
0021-9193/88/115208-08$02.00/0

Vol. 170, No. 11

lac Fusion Analysis of the *bet* Genes of *Escherichia coli*: Regulation by Osmolarity, Temperature, Oxygen, Choline, and Glycine Betaine

MARK W. ESHOO

Plant Growth Laboratory, University of California, Davis, California 95616

Received 31 December 1987/Accepted 12 August 1988

The synthesis of glycine betaine, a powerful osmoprotectant, from its precursor, choline, is a function of the *bet* genes. The *bet* genes code for the high-affinity transport of choline and the enzymes for its conversion to glycine betaine. These genes map at 7.5 min on the *E. coli* chromosome and are contained on the conjugative plasmid F'2. To study the transcriptional regulation of the *bet* genes in response to various environmental conditions, a collection of 30 *lac* operon fusions was isolated by utilizing the *bet* genes contained on F'2. Four osmoregulated *bet* loci (*betA*, *betB*, *betC*, and *betT*) were identified based on biochemical, regulatory, and merodiploid analysis of these fusions. All of the *bet* fusions demonstrated a 7- to 10-fold increase in transcription in response to increases in the osmotic strength of the growth medium. Choline further induced expression of *lac* fusions at the *betA*, *betB*, and *betT* loci when the cells were grown under conditions of osmotic stress. The end product of the pathway, glycine betaine, was a corepressor of choline induction for fusions at the *betA* and *betT* loci. Expression of the *betA*, *betB*, and *betT* loci was reduced 7- to 10-fold under anaerobic conditions. In addition, expression of the *betB* and *betT* loci was reduced when the cells were grown in high osmolarity at 16°C. These studies demonstrate that the expression of the *bet* genes is under the control of several environmental stimuli.

In recent years, knowledge of the molecular basis of adaptation to osmotic stress (i.e., osmoregulation) in bacteria such as *Escherichia coli* and *Salmonella typhimurium* has rapidly increased (5, 7–12, 19–22, 32, 37). These studies have shown that *E. coli* can adapt to environments of high osmolarity by the intracellular accumulation of compatible solutes. Depending on the growth conditions of the bacteria, these compatible solutes can include potassium (9, 18) trehalose (10, 20, 32), proline (5, 6, 8), and glycine betaine (19–22, 30). When such a molecule can be directly shown to protect the organism against inhibitory levels of osmotic stress, it is called an osmoprotectant. Glycine betaine, a powerful osmoprotectant (7, 21, 22), can be taken up from the environment or synthesized from its precursor, choline (19, 22, 33). The synthesis of glycine betaine from choline is a function of the *bet* activities (33). In *E. coli* the expression of the *bet* activities is controlled by the osmotic strength of the environment (19). These activities constitute one of the major responses to osmotic stress and confer high-level osmotic tolerance to the bacteria (19, 33).

The individual *bet* activities include the choline high-affinity transport system, choline dehydrogenase, and betaine aldehyde dehydrogenase. The *bet* genes map at 7.5 min on the *E. coli* chromosome and are fully complemented by the host genes carried on the conjugative plasmid F'2 (33).

Earlier studies demonstrated that the *bet* activities are induced under conditions of high osmolarity, can be further induced by the addition of choline to the growth medium, and require aerobic conditions for their expression (19, 33). Similar studies have shown that the glycine betaine periplasmic binding protein (*proU*) (3, 8), the trehalose phosphate synthase (*otsAB*) (10), and the outer membrane protein (*ompC*) (14–16) are also induced by high osmolarity and that this regulation is occurring at the transcriptional level.

In addition, the osmotically regulated outer membrane proteins (*ompF* and *ompC*) of *E. coli* have been shown to be regulated by the temperature of the growth medium (24, 25). Similarly, several other stress-related systems of *E. coli* have been shown to respond to more that one environmental stimulus. One of the best studied of these systems is the heat shock response of *E. coli* (28). These stress-induced proteins can be induced by several stimuli including temperature or alkaline shift (35), ethanol, and a variety of other chemical and physical inducers (28, 36).

To examine the *bet* region and its transcriptional regulation in response to various environmental stimuli, a collection of *lac* operon fusions was constructed with the *bet* genes contained on F'2. This analysis enabled us to identify the *betA* locus, first characterized by Styrvold et al. (33), in addition to three new osmoregulated *bet* loci, *betB*, *betC*, and *betT*. In this paper we show that, in addition to responding to exogenous choline and glycine betaine, expression of the *bet* genes is also regulated at the transcriptional level by the osmolarity of the growth medium, the temperature of the growth medium, and the presence of molecular oxygen in the growth medium.

MATERIALS AND METHODS

Bacterial strains and growth media. All bacterial strains are derivatives of *E. coli* K-12 (Table 1). A collection of *E. coli* SE5000 harboring *bet*::*lac* fusions on the conjugative plasmid F'2 was screened by replica plating onto plates of LOM2 medium containing 50 mg of 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal) per liter dissolved in dimethylformamide. LOM2 medium consists of 5 mM bistrispropane, 5 mM potassium phosphate, 10 mM ammonium sulfate, 0.5mg of $FeSO_4$ per liter, 1 mM $MgSO_4$, 0.05 mM thiamine, and 0.5% glycerol as the sole carbon source; 0.4 M NaCl was added to LOM2 medium for osmotica. The pH was adjusted to 7.0 with concentrated KOH. The ability to utilize choline as an osmoprotectant was determined by replica plating cells onto plates of medium 63 (26) containing 0.65 M NaCl and 1 mM

TABLE 1. Bacterial strains and bacteriophages

Strain or phage	Genotype	Source or derivation
E. coli		
MC4100	F⁻ *araD139* Δ(*argF-lac*)U169 *flbB5301 ptsF25 melA1 rpsL150 deoC1*	CGSC6152 B. J. Bachmann
SE5000	MC4100 *recA56*	G. Weinstock
SE5000R	SE5000 spontaneous rifampin resistance	This study
MBM7014	F⁻ *araC*(Am) *araD* Δ(*argF-lac*) U169 *trp*(Am) *mal*(Am) *rpsL relA thi supF*	G. Weinstock
AB247	F⁻ 2 (Bet⁺)/*sfa-Z relA1 spoT1 metB1*	CGSC247 B. J. Bachmann
ZIP514	F⁻ Δ *lac-3 trp-49 relA1 rpsL150 tsy-93 spoT1* Bet⁺	CGSC5948 B. J. Bachmann
MLE33Z	F'2 *betA5*::*lacZ lacY kan*/ZIP514	This study
MLE523Z	F'2 *betA8*::*lacZ lacY*, *kan*/ZIP514	This study
MLE32	F'2 *betA4*::*lacZ lacY*, *kan*/SE5000R	This study
MLE33	F'2 *betA5*::*lacZ lacY*, *kan*/SE5000R	This study
MLE54	F'2 *betA6*::*lacZ lacY*, *kan*/SE5000R	This study
MLE515	F'2 *betA7*::*lacZ lacY*, *kan*/SE5000R	This study
MLE523	F'2 *betA8*::*lacZ lacY*, *kan*/SE5000R	This study
MLE63	F'2 *betA9*::*lacZ lacY*, *kan*/SE5000R	This study
MLE720	F'2 *betA10*::*lacZ lacY*, *kan*/SE5000R	This study
MLE517	F'2 *betA11*::*lacZ lacY*, *kan*/SE5000R	This study
MLE410	F'2 *betB1*::*lacZ lacY*, *kan*/SE5000R	This study
MLE413	F'2 *betB2*::*lacZ lacY*, *kan*/SE5000R	This study
MLE424	F'2 *betB3*::*lacZ lacY*, *kan*/SE5000R	This study
MLE620	F'2 *betB4*::*lacZ lacY*, *kan*/SE5000R	This study
MLE67	F'2 *betT1*::*lacZ lacY*, *kan*/SE5000R	This study
MLE68	F'2 *betT2*::*lacZ lacY*, *kan*/SE5000R	This study
MLE74	F'2 *betT3*::*lacZ lacY*, *kan*/SE5000R	This study
MLE914	F'2 *betT4*::*lacZ lacY*, *kan*/SE5000R	This study
Phages		
λp*lac*Mu53	*imm* λ *'trp' lacZ*⁺ *lacY*⁺ *lacA 'uvrD xho*::*kan* Mu[*c*Its62 *ner*⁺*A*⁺ *'S*]	G. Weinstock
λpMu507	*c*Its857 *S*am7 Mu *A*⁺*B*⁺	G. Weinstock

choline and scoring for growth after 3 days. Merodiploids of the *bet* genes were constructed in *E. coli* ZIP514. This strain contains a small deletion of the *lac-codAB* region of the chromosome but still retains the *bet* activities (33). The *bet*::*lac* fusions contained on F'2 were introduced to this strain by conjugation. Transconjugates were isolated by selecting for the ability to utilize choline as an osmoprotectant and kanamycin resistance.

Osmotic induction of β-galactosidase in isolates with *bet*::*lac* fusions was determined by growing cells in media of differing osmolarities. Cultures grown at 0.06 osM were grown in LOM4 medium. LOM4 consists of 10 mM ammonium sulfate, 20 mM potassium phosphate monobasic, 0.5 mg of $FeSO_4$ per liter, 1.0 mM $MgSO_4$, and 0.2% glucose as the carbon source. The pH of this medium was adjusted to 7.0 with concentrated KOH.

Media of 0.160 osM consists of a 50% dilution of medium 63 (26) with the exceptions of the $MgSO_4$ and the $FeSO_4$. To obtain media with an osmolality of 0.30 osM and higher, NaCl was added to medium 63 containing 0.2% glucose, in 0.1 M increments, up to 0.5 M. *E. coli* SE5000R was used as the background strain to study the *bet*::*lac* fusions contained on the F' plasmids. Anaerobic conditions were prepared as follows: 125-ml flasks containing either 25 ml of medium 63 supplemented with 0.8% glucose and 0.3 M NaCl for osmotica or a 50% dilution of medium 63 were inoculated with *bet*::*lac* fusion strains. Flasks were then evacuated several times, and the air was flushed out with argon; flasks were then filled with argon and sealed.

Isolation of *bet*::*lac* fusions. A phage lambda derivative, λp*lac*Mu53, was employed for the construction of *lac* operon fusions with the *bet* promoters. This phage was chosen because it carries a separate gene for kanamycin resistance under the control of its own promoter and because, by the mechanism of Mu transposition, it is able to integrate randomly throughout the *E. coli* genome (2). To target the integration of these phage in the *bet* region, we utilized the conjugative plasmid F'2. F'2 contains approximately 0.8 min of the *E. coli* chromosome (13), with the *bet* genes being the only identified host genes contained on this plasmid (33). Isolation of λp*lac*Mu53 insertions in F'2 proved particularly useful for isolating *bet*::*lac* fusions, since some of the *bet* phenotypes cannot be readily selected and require an approach involving assaying for the individual activities.

To obtain insertions of the λp*lac*Mu53 in F'2, 1 ml of exponentially growing strain AB247 was infected with 1 ml of λp*lac*Mu53 containing approximately 10^8 PFU and 1 ml of λpMu507 helper phage stock containing approximately 10^9 PFU. After coinfection, cultures were incubated at 37°C in Luria broth containing 10 mM $MgSO_4$ for 30 min. Fresh Luria broth (5 ml) was added to the culture, and the cells were washed three times in Luria broth to remove any unattached phage. The cells were then filter mated with *E. coli* SE5000 on a 0.45-μm-pore-size membrane filter (Millipore Corp.) and incubated on a Luria broth plate for 60 min for the transfer of the F'2 containing λp*lac*Mu53 insertions. Single colonies of transconjugates, in which the phage had integrated into F'2, were selected on Luria broth plates containing 50 μg of kanamycin per ml and 50 μg of streptomycin per ml. These plates were then replica plated onto three different plates: LOM2 containing X-gal, LOM2 containing X-gal supplemented with 0.4 M NaCl, and medium 63 with 0.65 M NaCl and 1 mM choline. The first two LOM plates containing X-gal were used to identify any of the fusions that demonstrated some level of osmoregulation of β-galactosidase activity. The third plate of medium 63 containing 0.65 M NaCl and 1 mM choline was employed to identify any of the fusions which resulted in the loss of the ability to utilize choline as an osmoprotectant.

bet::*lac* fusions were transferred by conjugation from strain SE5000 to strain MC4100 and then to strain SE5000R. These transfers of the *bet*::*lac* fusions confirmed that the fusions had occurred on the F'2 and provided an effective way of separating the *bet*::*lac* fusions from any possible secondary λp*lac*Mu53 insertions in the chromosome. Three

constructions of the *bet*::*lac* fusions were performed to assure that several nonsibling *bet*::*lac* fusions were found for each locus.

Choline transport assays. The presence of the high-affinity choline transport activity was determined by the radiolabeled choline method of Styrvold et al. (33). The activity was determined as the rate of uptake of 10 μM [^{14}C]choline, defined as nanomoles per minute per milligram of protein.

Enzyme assays. All enzyme assays were carried out with saturating amounts of substrates (19). All activities are reported as nanomoles of product per minute per milligram of protein. Protein content was determined by the method of Lowry et al. (23). When whole cells were used in an enzyme assay, as in the β-galactosidase assays, cells were washed by centrifugation and suspended in ice-cold distilled water. This step was employed to remove from the medium any salts and glucose, which may have interfered with the protein determination or the β-galactosidase assay. Choline dehydrogenase activity was determined by the radiolabeled choline method of Landfald and Strom (19). In most cases toluene-treated cells were stored at −20°C and saved for betaine aldehyde dehydrogenase assays.

The NAD^+-dependent enzyme betaine aldehyde dehydrogenase was assayed spectrophotometrically. The enzyme reaction was measured by monitoring the rate of NADH production in a reaction mixture containing 90 mM sodium phosphate buffer (pH 7.5), 10 mM NAD, and 25 μl of crude extract. The reaction was initiated by the addition of 7 μl of a freshly prepared solution containing 100 mg of betaine aldehyde per ml. These reactions were carried out at room temperature, and the rate of NADH production was determined by measuring the optical density at 340 nm on a Varian/Cary 219 spectrophotometer with a plotter. Toluene-treated cells from the choline dehydrogenase assay were frozen overnight at −20°C. The freezing step is important to destroy any of the betaine aldehyde dehydrogenase activity of the membrane-bound choline dehydrogenase enzyme, as reported by Landfald and Strom (19). To prepare the crude extracts, cells were disrupted with a French press at 7,000 lb/in^2 and centrifuged for 30 min at 17,000 × *g*. The supernatant (crude extract) was then used in the reaction mixture. Note that Landfald and Strom (19) used a radioactive assay to determine the specific activity of the betaine aldehyde dehydrogenase activity, but the activities are comparable to those found with the spectrophotometric method described here.

β-Galactosidase activity in whole cells was determined as described by Miller (26). Cells were lysed with chloroform-sodium dodecyl sulfate (26). Since the cells were grown in various media affecting their optical densities and the osmolarity of the β-galactosidase reaction mixtures, all cells were suspended in distilled water before the assay for β-galactosidase activity. The amount of cellular protein was determined for each assay. The units of β-galactosidase activity are those defined by Miller (26).

RESULTS

Isolation of *bet*::*lac* fusions. To study the transcriptional regulation of the *bet* genes, a collection of 10,000 λp*lac*Mu53 insertions in the conjugative plasmid F′2 was isolated. This plasmid was employed as a means to target insertions in the *bet* region. From this collection, 29 cultures with osmoregulated *lac* operon fusions were isolated. These osmoregulated fusions were found at a relatively high frequency, approximately 10^{-2}, among those λp*lac*Mu53 insertions expressing β-galactosidase.

Eighteen of the isolates with osmoregulated fusions were chosen for study in detail because they resulted in the loss of one or more of the *bet* functions, as discussed below. The *lac* fusions were then used to study the transcriptional regulation of the *bet* genes in response to various environmental conditions. The 11 osmoregulated *lac* fusions resulting in normal *bet* functions may represent new classes of *bet* genes but were not studied further.

Characterization of the *bet*::*lac* fusions for the individual *bet* activities. It should be noted that relatively little information is yet available regarding the fine structure of the *bet* region, including the number of genes, promoters, operons, etc. Thus, a considerable amount of effort was devoted to phenotypic characterization of the individual fusions with the aim of building on this analysis for studies of the regulation of the *bet* gene promoters. It is these *bet*::*lac* fusions which provide an interesting opportunity for studying how environmental stimuli can affect gene expression (see below).

Analysis of the fusion collection has led to the identification of four osmoregulated *bet* loci: *betA*, *betB*, *betC*, and *betT*. Putative *bet*::*lac* fusions were scored for the various *bet* functions, including relief of osmotic inhibition of growth by choline, choline high-affinity transport activity, choline dehydrogenase activity, and betaine aldehyde dehydrogenase activity.

lac fusions at the *betA* locus in strain MLE33 resulted in the loss of choline dehydrogenase activity (Table 2); this represented a greater than 50-fold reduction of this activity. Furthermore, fusions at this locus resulted in an approximately twofold increase in both the choline high-affinity transport activity (11.5 nmol/min per mg of protein) and the betaine aldehyde dehydrogenase activity (67.6 nmol/min per mg of protein). The increase of the betaine aldehyde dehydrogenase activity was also observed by Styrvold et al. (33) in two of the three isolates with point mutations at the *betA* locus.

Fusions at the *betB* locus resulted in a loss of both the choline dehydrogenase and the betaine aldehyde dehydrogenase enzyme activities, but expression of the choline high-affinity transport was at wild-type levels (7.2 nmol/min per mg of protein). The choline high-affinity transport activity was greatly reduced in isolates with *betT* fusions (less than 8% of wild-type levels for strain MLE914), yet isolates with insertions at *betT* expressed both choline dehydrogenase and betaine aldehyde dehydrogenase at wild-type levels. Only isolates with *lac* fusions at the *betT* locus were able to utilize choline as an osmoprotectant under conditions of osmotic stress. It should be noted that *E. coli* SE5000R, although missing the choline high-affinity transport activity, still contained the choline low-affinity transport activity. The fourth osmoregulated *bet* locus, *betC*, was originally identified by the loss of all the *bet* functions. Southern blot hybridization experiments (data not shown) demonstrated that these strains contained deletions of the *betA*, *betB*, and *betT* loci. Growth of these strains was inhibited by choline, and strains that did not contain the deletions had an osmotically sensitive phenotype (data not shown). The possible significance of these findings is discussed below.

Effects of osmolarity. The range of osmolarities used to study the osmoregulation of the *bet*::*lac* fusions was 0.06 to 1.20 osM. *lac* fusions of the *bet* loci demonstrated osmotic regulation at the transcriptional level (7- to 10-fold induction), with maximal expression of the *bet*::*lac* fusions occurring when cells were grown in medium of 0.9 osM (Fig. 1). Each of the *bet* loci studied demonstrated osmoregulation at

TABLE 2. Scoring of the *bet* functions of *bet*::*lac* fusions

Strain	Relevant genotype	Osmoprotection by choline	Activity (nmol/min per mg of protein)		
			High-affinity choline uptake[a]	Choline dehydrogenase[b]	Glycine betaine aldehyde dehydrogenase[c]
MC4100 F'2	Wild type	+	7.7	6.8	36.0
MLE33	*betA5*	−	11.5	0	68.0
MLE32	*betA4*	−	8.5	0	76.0
MLE410	*betB1*	−	5.0	0	0
MLE413	*betB2*	−	7.2	0	0
MLE67	*betT1*	+	0.7	9.0	44.0
MLE914	*betT4*	+	0.9	10.8	40.0
MLE74	*betT3*	+	0.7	10.4	40.0

[a] Cells were grown to the midexponential phase in medium 63–glucose containing 0.5 M NaCl; rates were determined using a 10 μM concentration of ^{14}C-labeled choline.
[b] Enzymatic activities were assayed in toluene-treated cells grown in medium 63–glucose containing 0.4 M NaCl and 1 mM choline.
[c] Enzymatic activities were determined spectrometrically from crude extracts prepared from toluene-treated cells grown in medium 63–glucose containing 0.4 M NaCl and 1 mM choline.

the transcriptional level (Fig. 1). These increases of expression in response to increases of the osmotic strength of the growth medium resulted in maximal levels of β-galactosidase activity of 620 to 780 U. Induction of transcription by high osmotic strength was a general response and not a specific effect of sodium chloride, since equal osmolarities of potassium chloride and sucrose were equally effective in inducing expression of the *bet* genes. Several fusions at each loci were characterized and demonstrated regulation like that of the fusion strains shown (Fig. 1).

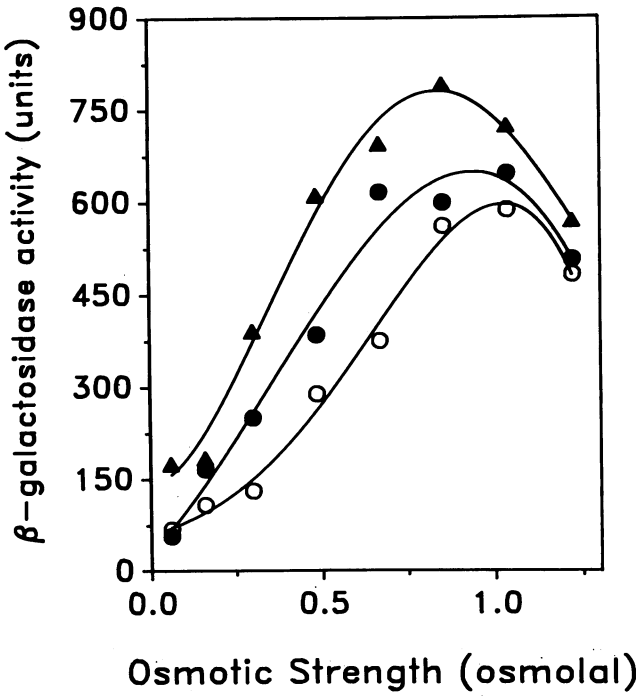

FIG. 1. Effects of the osmolarity of the growth medium on the transcription of *bet*::*lac* fusions. β-Galactosidase assays for cultures grown in medium of 0.060 osM were taken from cultures grown in medium LOM4. β-Galactosidase assays for cultures grown in medium of 0.160 osM were taken from cultures grown in a 50% dilution of medium 63. All media with osmolarity of 0.300 osM or above was medium 63–glucose with various amounts of NaCl added as osmotica. Symbols: ○, strain MLE33 (*betA*); ▲, strain MLE413 (*betB*); ●, strain MLE914 (*betT*).

Effects of osmoprotectants on osmotic regulation. The steady-state levels of β-galactosidase activity of fusions at the *betA*, *betB*, and *betT* loci grown under osmotic stress (0.90 osM) and 1 mM glycine betaine resulted in approximately 40% less expression (Table 3). Proline also reduced the steady-state levels for each of the *bet*::*lac* fusions, although not as much as glycine betaine. For example strain MLE33, with a fusion at the *betA* locus, expressed β-galactosidase activity of 560 U when grown in medium of high osmotic strength (0.90 osM). The same strain with a *betA* fusion grown in the same medium expressed β-galactosidase activity of 370 and 450 U with the addition of 1 mM glycine betaine or 1 mM proline, respectively. As is the case with most other genes in *E. coli* that are induced by medium of high osmotic strength, relief of osmotic stress by glycine betaine results in the reduction of their respective levels of osmotic induction. This finding supports the idea that there is a common mechanism of osmoregulation of gene expression in *E. coli* (see below).

Choline regulation of the *betA*, *betB*, and *betT* loci. Landfald and Strom (19) found that the addition of choline to osmotically stressed cells of *E. coli* resulted in increased levels of activity of the enzymes in the choline-to-glycine betaine pathway. To determine whether this induction was occurring at the transcriptional level, the effects of choline on osmotically stressed *bet*::*lac* fusions were studied.

Clearly, expression of *betA*, *betB*, and *betT* loci demonstrated an additional three- to fourfold increase of transcription during a 3-h choline induction assay (Fig. 2). For

TABLE 3. Effects of the osmoprotectants, glycine betaine, and proline on steady-state expression of *bet*::*lac* fusions[a]

Strain	Relevant genotype	β-Galactosidase activity (U) with:		
		No addition	Glycine betaine	Proline
MLE33	*betA5*	560	370	450
MLE32	*betA4*	470	230	285
MLE413	*betB2*	790	573	620
MLE410	*betB1*	610	410	470
MLE74	*betT3*	470	320	400
MLE914	*betT4*	600	380	430

[a] Cells were grown to the midexponential phase in medium 63–glucose containing 0.3 M NaCl and 1 mM concentration of osmoprotectant at 37°C, and β-galactosidase activity was assayed.

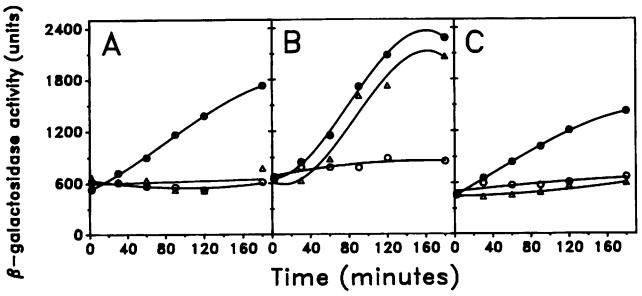

FIG. 2. Effect of the addition of 1 mM choline and the addition of 1 mM choline plus 1 mM glycine betaine on the expression of exponentially growing *bet*::*lac* fusion strains. Cultures were grown in medium 63–glucose containing 0.3 M NaCl (0.850 osM) to an approximate density of 80 μg of protein per ml. At time zero 1 mM choline or 1 mM choline plus 1 mM glycine betaine was added. At 2, 30, 60, 90, 120, and 180 min, samples of the growing cultures were taken. Cell growth was stopped by placing samples in prechilled test tubes in an ice bath. Cells were pelleted by centrifugation and suspended in ice-cold distilled water, and β-galactosidase was assayed. (A) Strain MLE33 (*betA*); (B) strain MLE413 (*betB*); (C) strain MLE914 (*betT*). Symbols: ○, medium 63–0.3 M NaCl; ●, medium 63–0.3 M NaCl supplemented with 1 mM choline; △, medium 63–0.3 M NaCl supplemented with 1 mM choline and 1 mM glycine betaine.

example, fusions at the *betB* locus resulted in an increase from 640 to 2,300 U of β-galactosidase 3 h after the addition of choline to cells grown under high osmolarity. Comparable increases were also seen with fusions at the *betA* and the *betT* loci (Fig. 2A and C). The addition of betaine aldehyde or glycine betaine did not induce transcription of any of the *bet*::*lac* fusions.

Effects of glycine betaine on choline regulation of the *betA*, *betB*, and *betT* loci. To study the effects of glycine betaine on choline induction of the *betA*, *betB*, and *betT* loci, glycine betaine (1 mM) was added simultaneously with choline (1 mM) during induction experiments. Interestingly, the presence of 1 mM glycine betaine in the growth medium repressed choline induction of fusions of the *betA* and *betT* loci but not the *betB* locus (Fig. 2B). Repression of choline induction was specific with glycine betaine; betaine aldehyde, proline, and several amino acids when added together with 1 mM choline did not inhibit the choline induction. For example, after a 3-h choline induction assay, fusions at the *betA* locus demonstrated an increase of β-galactosidase activity from 560 to 1,700 U. The simultaneous addition of 1 mM glycine betaine and 1 mM choline resulted in the near-basal level of β-galactosidase activity of 770 U after the 3-h assay. When 1 mM proline was added together with 1 mM choline, *betA* fusions expressed β-galactosidase at 1,700 U at the end of the 3-h assay (data not shown). Choline induction of the *betB* locus is not repressed by the presence of 1 mM glycine betaine in the growth medium. After the 3-h induction assay, an increase from 630 to 2,100 U was observed when 1 mM choline and 1 mM glycine betaine were added together, compared with 2,300 U when choline was added alone.

Effects of anaerobic conditions. Landfald and Strom (19) reported earlier that cells grown under anaerobic conditions are not protected from osmotic stress by choline, and that the levels of the enzymes activities in the *bet* pathway are markedly reduced under these growth conditions. *lac* fusions at the *betA*, *betB*, and *betT* loci displayed a 7- to 10-fold reduction of β-galactosidase activity in when the cells were grown anaerobically under conditions of osmotic stress (0.90 osM) (Table 4). For example, fusions at the *betT* locus characterized by strain MLE914 exhibited β-galactosidase activity of 69 U when grown in medium of 0.90 osM under anaerobic conditions compared with 600 U of activity when the cells were grown aerobically. Fusions grown under low osmolality (0.16 osM) and anaerobiosis demonstrated even lower levels of β-galactosidase, suggesting that these two effects are additive.

TABLE 4. Effects of oxygen in the growth medium on the expression of *bet*::*lac* fusions

Strain	Relevant genotype	β-Galactosidase activity (U)			
		Anaerobic[a]		Aerobic[b]	
		Low osm	High osm	Low osm	High osm
MLE33	*betA5*	10	120	115	560
MLE32	*betA4*	20	90	90	470
MLE413	*betB2*	20	160	185	790
MLE410	*betB1*	50	120	200	610
MLE914	*betT4*	10	85	165	600
MLE74	*betT3*	25	110	100	610

[a] Anaerobic cultures were grown to the midexponential phase at 37°C in sealed flasks containing an atmosphere of pure argon, and β-galactosidase was assayed. Low-osmolarity (osm) cultures were grown in a 50% dilution of medium 63 supplemented with 0.8% glucose. High-osmolarity cultures were grown in medium 63 supplemented with 0.3 M NaCl and 0.8% glucose.

[b] Aerobic cultures were grown to the midexponential phase at 37°C. Low-osmolarity cultures were grown in a 50% dilution of medium 63 containing 0.2% glucose. High-osmolarity cultures were grown in medium 63 supplemented with 0.3 M NaCl and 0.2% glucose.

Temperature regulation of the *bet* genes. Low growth temperatures reduced the levels of β-galactosidase activity of fusions in the *betB* and *betT* loci (Table 5). Isolates with fusions at these loci, when grown under conditions of osmotic stress and low temperature (16°C), demonstrated a fivefold reduction of the β-galactosidase activity when compared with cultures grown at 37°C. Cultures of strain MLE413 (*betB*::*lac*) grown at 16°C in medium of 0.90 osM expressed β-galactosidase activity of 170 U, compared with 790 U of activity when the cells were grown in the same medium at 37°C. Inhibition of expression by low temperature for the *betB* and *betT* loci was found to occur in the 22 to 18°C range and could not be detected above 24°C. Due to problems of instability of λp*lac*Mu53 insertions associated with high temperatures, only the effects of low temperature were studied.

Interestingly, fusions at the *betA* locus were not temperature regulated. Merodiploids were constructed to test whether the *betA* locus is normally temperature regulated, but *lac* fusions at this locus are not due to some lost gene product. Merodiploids of the *betA* fusions were temperature regulated (Table 5). The results of this analysis demonstrate that the *betA* locus can be temperature regulated when a second copy of the *bet* genes is present. Furthermore, analysis of the merodiploids demonstrated that the loss of temperature regulation can be complemented in *trans*. In summary, these experiments demonstrate that the expression of the *bet* genes is regulated by the temperature of the growth medium.

DISCUSSION

Gene fusion analysis has proven to be a particularly useful tool for the study of a wide variety of systems that respond to environmental stimuli. In this study, the phage lambda

TABLE 5. Effects of temperature on the expression of *bet*::*lac* fusions[a]

Strain	Relevant genotype	β-Galactosidase activity (U)			
		16°C		37°C	
		Low osm	High osm	Low osm	High osm
MLE32	*betA4*	85	700	90	500
MLE33	*betA5*	75	740	115	560
MLE33Z	*betA5*/*betA*$^+$	87	165	120	470
MLE413	*betB2*	77	160	183	790
MLE410	*betB1*	83	180	200	610
MLE914	*betT4*	120	110	165	600
MLE74	*betT3*	100	160	100	470

[a] Cultures were grown aerobically to the midexponential phase at 16°C. Low-osmolarity (osm) cultures were grown in a 50% dilution of medium 63 containing 0.2% glucose. High-osmolarity cultures were grown in medium 63 supplemented with 0.3 M NaCl and 0.2% glucose.

derivative λp*lac*Mu53 was used for two purposes: as a vehicle to form stable insertion mutations and as a tool to study the transcriptional regulation of the gene at the site of integration. All of the *bet* loci characterized in this study resulted in the loss of one or more of the *bet* activities, thereby yielding a collection of insertion mutants of individual *bet* genes. Analysis of the phenotypes and regulation of the *bet* fusion collection resulted in the identification and characterization of four osmoregulated *bet* loci: *betA*, *betB*, *betC*, and *betT*. It should be noted that approximately 40% of our osmoregulated *bet*::*lac* fusion collection did not result in the loss of any *bet* functions. Thus, it is interesting to speculate that there are several other osmoregulated loci in this region which are involved in some unknown aspect of the *bet* functions, possibly regulation.

Whereas the mechanism of coupling environmental stimuli such as osmolarity to gene expression is not yet fully understood, some pertinent points are worth mentioning. Laimins et al. (18) have shown that the high-affinity potassium transporter, the *kdp* operon, is regulated by changes in turgor pressure. Sutherland et al. (17, 34) have recently proposed that the osmotically regulated *proU* locus responds to the intracellular potassium concentration, which changes in response to changes in the osmotic strength of the growth medium. They showed that the accumulation of glycine betaine and other osmoprotectant compounds reduces the intracellular concentration of potassium. It is this reduction in the intracellular levels of potassium that is believed to result in decreased expression of the *proU* locus (34). The presence of glycine betaine in the growth medium also affected the expression of the outer membrane proteins of *E. coli*. Barron et al. (1) have also shown that the well-characterized pattern of the outer membrane proteins *ompF* and *ompC* in response to high osmotic strength is reversed by the presence of 1 mM glycine betaine in the growth medium. As observed for the osmoregulated *proU*, *ompF*, and *ompC* loci, glycine betaine reduced osmotic induction of the *bet* genes. Therefore, it seems possible the *bet* genes shared a common mechanism for osmoregulation of gene expression, although it is not known whether pleiotropic genes are involved or whether each of these systems is responding independently to the same signal.

In addition to being osmoregulated, expression of the *betA*, *betB*, and *betT* promoters can be further induced by the presence of choline in the growth medium. For example, the addition of 1 mM choline to cultures growing in high osmolarity medium results in a three- to fivefold increase in expression of the *betA*, *betB*, and *betT* loci. Only choline was able to induce the *bet* genes; betaine aldehyde, proline, and glycine betaine did not induce expression of any of the fusions. In fact, glycine betaine was found to be a corepressor of choline induction for fusions at the *betA* and *betT* loci, blocking all induction by choline. This corepression of choline induction by glycine betaine is different from the above-mentioned effect of proline and glycine betaine on the osmoregulation of the *bet* loci in that only glycine betaine demonstrated this ability. Furthermore, only fusions at the *betA* and *betT* loci demonstrated corepression by glycine betaine on choline induction; fusions at the *betB* locus were induced by choline, even in the presence of glycine betaine in the growth medium. These data demonstrate that *E. coli* has a specific and distinct mechanisms for the detection of glycine betaine and choline in the growth medium, and the presence of these compounds can affect gene expression.

In addition to the effects of osmolarity and metabolites of the pathway, we examined the effects of several other environmental conditions on the regulation of the *bet* genes. The availability of oxygen and low growth temperatures were found to have the most pronounced effects on the regulation of the *bet* genes. The synthesis of glycine betaine from choline is a oxidative process which requires the presence of molecular oxygen as a substrate (19). In the absence of oxygen, choline cannot be converted to glycine betaine and has no osmoprotective properties of its own. Landfald and Strom (19) have shown that the *bet* enzyme activities are greatly reduced when the cells are cultured anaerobically. In agreement with this is the finding that transcription of the *betA*, *betB*, and *betT* loci is greatly reduced under anaerobic conditions.

Expression of the *bet* genes is also regulated by the temperature of the growth medium. Expression of fusions at the *betB* and *betT* loci was inhibited in medium of high osmotic strength when the cells were grown at low temperatures. Expression of fusions at the *betA* locus was not repressed under conditions of low growth temperature. Merodiploid analysis of fusions at this locus demonstrated restored temperature regulation. The finding that temperature regulation can be complemented in *trans* demonstrates that this regulation is a specific function of the *bet* genes and not just the result of reduced expression of β-galactosidase at low temperature.

Growth in medium of high osmotic strength and low temperature also results in a pattern of expression of the *ompF* and *ompC* like that found in non-osmotically stressed cells (24). Lundrigan and Earhart (25) identified the *envY* gene, which is responsible for thermoregulation of the osmoregulated porins *ompF* and *ompC*, although it is not known whether this gene is responsible for the temperature regulation of the *bet* genes.

Due to the identification of secondary mutations in strains containing *betC*::*lac* fusions, biochemical and regulatory analyses were not performed on these strains. *betC*::*lac* fusion strains were unusual in that all strains were found to contain deletions of the *betA*, *betB*, and *betT* loci, and the presence of the other *bet* loci in these strains resulted in a osmotically sensitive phenotype. This finding suggests that there was a selective pressure for isolates containing the deletions. Indeed, osmoregulation is central to bacterial growth and physiology, and many mutations that affect osmoregulation may be lethal to the cell. Mutations at the *betC* locus may be of this type. Future analysis of λ transductants of these fusions, which are diploid and contain a functional *betC* gene, may provide a way of overcoming

the osmosensitive phenotype and help define the mechanism of osmotic and choline regulation of the *bet* genes.

In this paper, we have demonstrated that several environmental conditions can affect the expression of the *bet* genes of *E. coli*. Several other researchers have identified stress responses that are regulated by multiple environmental conditions. It has been shown by Christman et al. that the hydrogen peroxide inducible genes overlap with the heat shock proteins (4, 27), and it is also clear that several other stresses can affect the heat shock response, including an alkaline shift (35) and heavy metals and ethanol (28, 36). It has also been shown that an alkaline shift is able to induce the SOS functions in *E. coli* (31). Nixon et al. (29) have proposed a molecular basis for an integration of stress-related regulons as they have demonstrated that there are sequence homologies among a variety of regulatory systems that control responses to environmental stresses including the osmoregulatory genes *ompR* and *envZ*.

In summary, expression of the *bet* genes, in addition to being regulated at the transcriptional level by osmotic stress, is also regulated by the presence of choline and glycine betaine, low temperature stress, and the availability of molecular oxygen. Future work in the fine-structure mapping of the *bet* genes and DNA sequencing of the promoters should shed light on the mechanism by which several environmental stimuli can regulate gene expression.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant GM 34912 from the National Institute of Health.

Special thanks go to R. C. Valentine, L. T. Smith, and D. Ran (University of California, Davis) and A. R. Strom (University of Tromso, Norway) for useful discussions and editing of the manuscript.

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