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# Integration Host Factor Is Required for Positive Regulation of the *tdc* Operon of *Escherichia coli*

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**A 14-bp segment in the promoter region of the *tdcABC* operon of *Escherichia coli* shows sequence identity with the consensus binding site for the *E. coli* integration host factor (IHF). In an *himA* (IHF-deficient) strain, expression of β-galactosidase from a *tdcB'-'lacZ* protein fusion plasmid was about 10% of that seen with an isogenic *himA*$^+$ strain. Threonine dehydratase activity from the chromosomal *tdcB* gene in the *himA* mutant was also about 10% of the wild-type enzyme level. Two different mutations introduced into the putative IHF-binding site in the fusion plasmid greatly reduced the plasmid-coded β-galactosidase activity in cells containing IHF. In vitro gel retardation and DNase I footprinting analyses showed binding of purified IHF to the wild-type but not to the mutant promoter. IHF protected a 31-bp region between −118 and −88 encompassing the conserved IHF consensus sequence. These results suggest that efficient expression of the *tdc* operon in vivo requires a functional IHF and an IHF-binding site in the *tdc* promoter.**

Biodegradative threonine dehydratase (EC 4.2.1.16) of *Escherichia coli* catalyzes the pyridoxal phosphate-dependent dehydration of L-threonine and L-serine to ammonia and to α-ketobutyrate and pyruvate, respectively. The enzyme is induced under anaerobic culture conditions in tryptone yeast extract (TYE) medium or in a defined synthetic medium (H4) containing four amino acids (threonine, serine, valine, and isoleucine) plus cyclic AMP and fumarate (16). A 6.3-kb *E. coli* DNA fragment, cloned and sequenced in this laboratory, contains the dehydratase structural gene, *tdcB*, as part of a polycistronic operon, *tdcABC* (5, 13, 14). The *tdcC* gene encodes a membrane-associated threonine-serine permease (33), and the *tdcA* gene product appears to act as a *trans*-acting positive activator for autogenous regulation of the *tdc* operon (26). Efficient expression of the *tdc* operon is also positively regulated by the product of a regulatory gene, *tdcR*, located immediately upstream of *tdcABC* in opposite transcriptional orientation (30).

The 234-bp untranslated region between the *tdcR* and *tdcA* reading frames constitutes the *tdc* promoter in tandem with the *tdcR* promoter (Fig. 1). The nucleotide sequence shows recognizable −10 and −35 sequences typical of *E. coli* promoters and the purine-rich Shine-Dalgarno sequences for ribosome binding. The S1 nuclease mapping data revealed *tdc* transcription start sites at two adjacent nucleotides, designated +1 at G, 32 nucleotides upstream of the *tdcA* start codon (14). The intercistronic region also contains sequences bearing significant homologies to the consensus binding sites for the catabolite gene activator protein (CAP) at around −45 and the *E. coli* integration host factor (IHF) near −110 (Fig. 1). Because expression of the *tdc* operon in vivo requires cyclic AMP (16), it is not surprising to find a CAP site in the *tdc* promoter; presumably, like other cyclic AMP-dependent systems, binding of cyclic AMP-CAP to this site is needed for transcriptional activation of the *tdc* genes. However, the significance of the putative IHF sequence in the *tdc* promoter as yet remains unknown. Recent evidence indicates that IHF, a heterodimeric protein of 20,000 Da encoded by the *himA* and *himD* genes, is involved in several cellular processes, including lambda site-specific recombination, plasmid maintenance and transfer, and some bacterial gene expression (reviewed in reference 9). For example, expression of isoleucine-valine enzymes from the *ilvGMEDA* and *ilvBN* operons of *E. coli* is drastically reduced in *him* mutants (8, 9), and in vitro gel shift and footprinting assays showed that IHF binds specifically to the upstream promoter regions of *ilvGMEDA* (23, 24, 36) and *ilvBN* (35). On the other hand, IHF represses transcription from a *Chlamydomonas* chloroplast promoter in *E. coli* in vivo; DNase I and methylation protection experiments revealed binding of IHF to a site within the chloroplast promoter (34). To determine the functionality of the IHF site in the *tdc* promoter, we compared β-galactosidase activities from *tdcB'-'lacZ* fusions containing the wild-type and two mutant IHF sequences in *himA*$^+$ and *himA* strains. The results showed decreased transcription of the *tdc* operon in the absence of a functional IHF; further, mutations in the IHF site also reduced β-galactosidase expression from *tdcB'-'lacZ* in an IHF-positive host. In vitro gel retardation and footprinting experiments revealed IHF binding to the wild-type, but not to the mutant, IHF consensus sequence in the *tdc* promoter. These data suggest positive regulation of the *tdc* operon by IHF.

## MATERIALS AND METHODS

**Materials.** Tryptone, yeast extract, and other medium components were purchased from Difco Laboratories (Detroit, Mich.). Antibiotics and other reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.). DNA polymerase (Klenow), T4 DNA ligase, and restriction enzymes were supplied by Bethesda Research Laboratories (Gaithersburg, Md.) or Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Sequenase (T7 DNA polymerase) was a product of United States Biochemicals (Cleveland, Ohio). α-$^{35}$S-dATP (specific activity, >3,000 Ci/mmol) was bought from Amersham Corp. (Arlington Heights, Ill.). Purified IHF, prepared as described previously (21), was a generous gift from Howard Nash.

**Bacterial strains and growth media.** *E. coli* MC4100 [F$^-$ *araD139* Δ(*argF-lac*) *U169 rpsL150 deoC1 relA1 rbsR*

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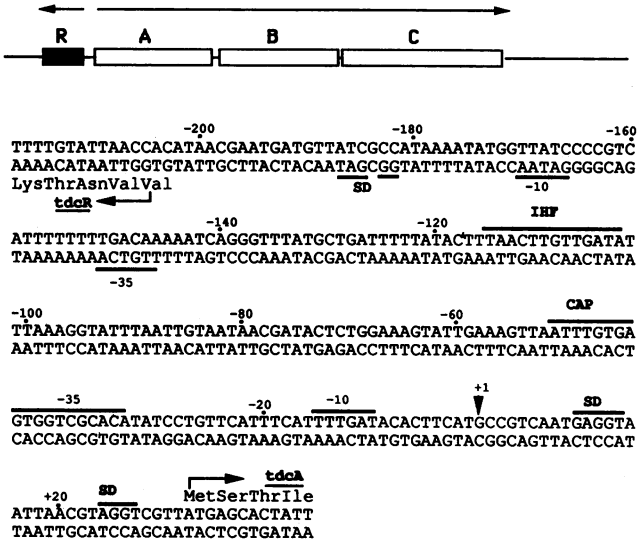


FIG. 1. Organization of the *tdc* operon and nucleotide sequence of the intercistronic region between *tdcR* and *tdcA*. The nucleotide number is assigned with reference to the transcription start site (+1) of the *tdc* operon. The putative −10 and −35 regions and the Shine-Dalgarno (SD) sequences are marked. The overlined sequences designated IHF and CAP represent consensus binding sites for IHF and CAP, respectively.

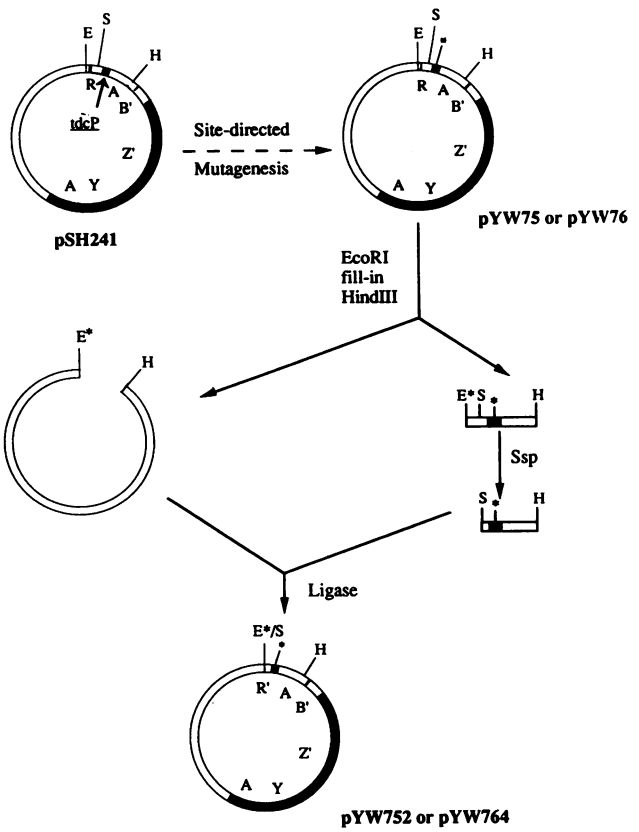


FIG. 2. Scheme for plasmid construction. pYW75 and pYW76 are *tdcR*$^+$ *tdcAB'-'lacZYA* fusions harboring a substitution and a deletion, respectively, in the putative IHF binding site in the *tdc* promoter (*tdcP*) as marked by an asterisk. pYW752 and pYW764 are *tdcR*-minus derivatives of pYW75 and pYW76, respectively. Abbreviations for restriction sites are as follows: E, *Eco*RI; E*, filled-in *Eco*RI; S, *Ssp*I; H, *Hin*dIII. For details, see Materials and Methods.

*ptsF25 flbB5301*] and DH5αF' [ϕ80 *d lacZ* ΔM15 Δ(*lacZYA-argF*) *U169 recA1 endA1 hsdR17* ($r_K^-$ $m_K^+$) *supE44* λ$^-$ *thi-1 gyrA relA1*] (33) were used for the propagation and maintenance of plasmids or as a host for phage M13. Strain CJ236 [*dut-1 ung-1 thi-1 relA1* (pCJ105) ($Cm^r$)] (18) was employed for the generation of uracil-substituted DNA. Strain HP4110 [MC4100 *himA* (P1) ($Tet^r$)] was obtained by the P1 transduction of *himA*::Tn*10* from strain K1299 (10) and by selecting for tetracycline resistance. Strain TH274 (MC4100 Δ *tdc-274 zgi-1*::Tn*10*) was constructed by a procedure identical to that described for strain TH16 (MC4100 Δ *tdc-216 zgi-1*::Tn*10*) (29), except that the deletion of *tdc* DNA extended from $BclI_{1447}$ (codon 72 of *tdcR*) to $BclI_{4570}$ (codon 206 of *tdcC*) (31). LB, YT, and M9 minimal media were prepared as described by Miller (20) or Sambrook et al. (27). The synthetic H4 medium was that of Hobert and Datta (16). The TYE medium contained 2% tryptone and 1% yeast extract supplemented with salts and pyridoxine hydrochloride (16).

**Plasmids.** The *tdcB'-'lacZ* fusion plasmids pSH240 (*tdcR*) and pSH241 (*tdcR*$^+$) have been previously described (30). pYW90, a *tdcR'-'lacZ* fusion plasmid, was obtained by isolating a 578-bp *Hin*cII-*Hpa*I (nucleotides [nt] 1424 to 2001 [31]) fragment from pSH241 containing the first 82 codons of *tdcR* and ligating it to *Sma*I-cleaved pMC1403 DNA (3). The fusion, which had the entire *tdcR-tdcA* promoter sequence and the 32 codons of the N-terminal end of *tdcA*, was characterized by restriction digest and DNA sequence analysis. pYW75 and pYW76 were constructed by site-directed mutagenesis of pSH241 harboring, respectively, base substitutions and deletion of the putative IHF site in the *tdc* promoter (see below). pYW752 and pYW764 were *tdcR*-minus derivatives of pYW75 and pYW76, respectively, and were constructed as shown in Fig. 2. Briefly, pYW75 (or pYW76) was digested with *Eco*RI, and the 3' recessed ends were filled in with Klenow DNA polymerase. The cleaved plasmid was further digested with *Hin*dIII to yield 1.1- and 10.5-kb fragments. The purified 1.1-kb fragment was treated with *Ssp*I, and the isolated 0.85-kb *Ssp*I-*Hin*dIII fragment was subsequently ligated to the purified 10.5-kb *Eco*RI (blunt)-*Hin*dIII fragment to yield pYW752 (or pYW764). To construct pYW2410 and pYW750, the source of DNA fragments for DNase I footprinting, purified *Eco*RI-*Hin*dIII fragment from pSH241 (or pYW75) was digested with *Hgi*AI, and the isolated 700-bp fragment was treated with T4 DNA polymerase and all four deoxynucleoside triphosphates to fill in the recessed ends. The fragment was further digested with *Sau*3AI to yield a 458-bp *Sau*3AI-*Hgi*AI (blunt) fragment extending from nt 1447 to 1905 (31), and it was then cloned into pUC19 previously cleaved with *Bam*HI and *Hin*cII.

**Site-directed mutagenesis.** To isolate mutants having base substitutions or deletion in the putative IHF consensus sequence, we used the protocol of Kunkel et al. (18). Briefly, the 1.1-kb *Eco*RI-*Hin*dIII fragment from pSH241 was cloned into M13mp19, and single-stranded uracil-containing DNA was isolated by employing strain CJ236 (18). Two synthetic oligonucleotides were used as mutagenic primers: (i) 5' CTTTAGCTTGTCGACATTTAA 3', from nt −118 to −98 on the *tdc* promoter (Fig. 1), with base substitutions A→G at −113 and T→C at −107 and at −104 which yielded a new *Sal*I site; and (ii) 5' TTTATGCTGATATCTATATTAAAG GTATTTA 3', from nt −136 to −89, with a 17-bp deletion between nt −118 and −102 and with additional substitutions

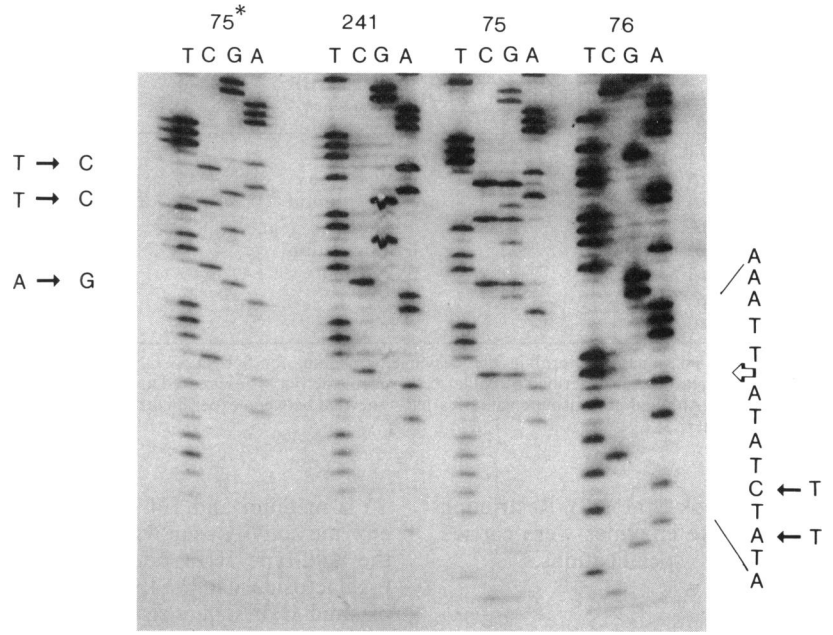


FIG. 3. A segment of sequencing gel depicting alterations in the nucleotide sequence of the mutant plasmids. The numbers at the top correspond to plasmids pSH241, pYW75, and pYW76. The base substitutions introduced into pYW75 are indicated at the left of the figure. The sequence of the deletion mutation in pYW76, the fusion junction (marked by an open arrow), and the two base substitutions are shown at the right. The dideoxy sequencing reactions contained the standard deoxynucleoside triphosphates except in 75*, in which dITP was used to minimize band compression.

T→A at nt −125 and T→C at −123 to create an *Eco*RV restriction site. After annealing to the single-stranded DNA, the extension reaction was carried out with T7 DNA polymerase followed by transfection of strain DH5αF′. The phage DNA from purified plaques was screened by digesting it with appropriate restriction enzymes, and the predicted mutations were confirmed by dideoxy sequencing (28) of the phage-derived, single-stranded DNA by using the Sequenase kit supplied by United States Biochemicals (Fig. 3). Finally, the mutant 1.1-kb *Eco*RI-*Hin*dIII fragments containing the substitution and deletion in the IHF consensus sequence in the *tdc* promoter were excised from the replicative form of M13mp19 and cloned into the corresponding *Eco*RI and *Hin*dIII sites in pSH241 to yield pYW75 and pYW76.

**Gel mobility shift assay.** Gel mobility shift assays were carried out essentially as previously described (15), with some minor modifications. For the binding reaction, the 354-bp *Dde*I-*Hpa*I fragments from pSH241 and pYW75, extending from nt −226 to +129 (from codon 8 of *tdcR* to codon 32 of *tdcA*), were end labeled by filling in with Klenow DNA polymerase and α-$^{35}$S-dATP. The labeled fragments (10 ng) were separately incubated with cell extracts (10 to 20 μg of protein) of strain MC4100 (*himA*$^+$) or HP4110 (*himA*) or with 10 to 20 ng of purified IHF at 22°C for 20 min in a 25-μl reaction mixture containing 50 mM Tris-HCl (pH 7.5), 7 mM KCl, 1.1 mM EDTA, 1 mM dithiothreitol, 7 mM $MgCl_2$, 3 mM $CaCl_2$, 10% glycerol, 200 μg of nuclease-free bovine serum albumin per ml of reaction mixture, and 0.5 μg of denatured, sonicated herring sperm DNA per ml. After incubation, the samples were electrophoresed at 22°C through a 4% native polyacrylamide gel for 1 to 2 h at 350 V in running buffer composed of 0.045 M Tris, 0.045 M boric acid, and 1 mM EDTA. The gels were then dried under vacuum and exposed to Kodak X-AR5 film to visualize the bands by autoradiography.

**DNase I footprinting.** We adopted the method of Galas and Schmitz (11) as modified for IHF footprinting by Craig and Nash (4). To label the 3′ end of the noncoding strand, the 457-bp *Sma*I-*Hin*dIII fragment from pYW2410 (wild-type DNA) or pYW750 (mutant DNA) was filled in at the *Hin*dIII end with α-$^{35}$S-dATP and unlabeled dGTP, dCTP, and dTTP. For end labeling the coding strand, a similar filling-in reaction was carried out at the *Dde*I end of the 354-bp *Dde*I-*Hpa*I fragment isolated from pSH241 or pYW75. The labeled DNA fragments (0.05 pmol) were separately incubated for 15 min at 22°C with and without purified IHF (20 ng) in a total volume of 50 μl of the binding buffer (4). After incubation, the mixtures were treated with 5 ng of freshly diluted DNase I (Sigma) for 90 s at 22°C. Digestion was stopped by adding an equal volume of a stop solution containing 0.6 M ammonium acetate, 0.1 M EDTA, and 50 μg of sonicated herring sperm DNA per ml. The samples were then extracted with phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol) and precipitated twice with ethanol. The precipitates were dissolved in a formamide-dye mixture (95% formamide, 20 mM EDTA, 0.05% bromophenol, 0.05% xylene cyanole FF) and electrophoresed through an 8% polyacrylamide–8 M urea sequencing gel in 0.5× Tris-borate-EDTA buffer (27). The A > C sequencing reaction of the same DNA fragments was performed by an alternate method of the Maxam-Gilbert sequencing protocol (2).

**Enzyme induction and assay.** Cultures were incubated anaerobically without shaking (still culture) in TYE or H4 medium for 8 h at 37°C. The threonine dehydratase activity of washed, toluene-treated cells was assayed colorimetrically as described previously (16). Specific activity is expressed as nanomoles of α-ketobutyrate formed per minute per milligram of protein. β-Galactosidase activity in sodium dodecyl sulfate-chloroform-permeabilized cells was measured as described by Miller (20), and specific activity is expressed as milliunits per minute per milligram of protein.

**Other methods.** The general techniques of DNA manipu-

TABLE 1. Requirement of functional IHF for TdcB-LacZ expression

| Strain and plasmid | Relevant genotype | Plasmid IHF sequence | LacZ sp act (%) with[a]: | |
|---|---|---|---|---|
| | | | TYE | H4 |
| MC4100(pSH241) | *himA*$^+$/*tdcB*::*lacZ* | Wild type | 5,198 (100) | 9,052 (100) |
| HP4110(pSH241) | *himA*/*tdcB*::*lacZ* | Wild type | 457 (9) | 931 (10) |
| MC4100(pYW75) | *himA*$^+$/*tdcB*::*lacZ* | Substitution | 645 (12) | 759 (8) |
| MC4100(pYW76) | *himA*$^+$/*tdcB*::*lacZ* | Deletion | 1,697 (33) | 1,993 (22) |
| HP4110(pYW75) | *himA*/*tdcB*::*lacZ* | Substitution | 149 (3) | 688 (7) |
| HP4110(pYW76) | *himA*/*tdcB*::*lacZ* | Deletion | 772 (15) | 2,378 (26) |
| MC4100(pMC1403) | *himA*$^+$/vector | | 58 (1) | 60 (1) |
| HP4110(pMC1403) | *himA*/vector | | 132 (3) | 301 (3) |

[a] Cultures were incubated anaerobically (stationary culture) in the designated medium for 8 h at 37°C. β-Galactosidase activity in sodium dodecyl sulfate-chloroform-permeabilized cells was measured as described by Miller (20); specific activity is expressed as milliunits per minute per milligram of protein. The assays were repeated at least four times. Although the absolute values varied somewhat between experiments, the relative values (in parentheses; expressed as percentages) were within 10% of each other.

lations were as outlined by Sambrook et al. (27). Restriction digestions of DNA with appropriate enzymes were carried out according to the manufacturers' specifications.

## RESULTS

**Requirement of IHF for *tdc* expression.** pSH241 is a *tdcB'-'lacZ* protein fusion plasmid in which codon 172 of *tdcB* was fused in frame to codon 8 of *lacZ* to produce a hybrid TdcB'-'LacZ from the *tdc* promoter (30). The plasmid also contains complete *tdcA* and *tdcR* genes. When incubated anaerobically in TYE medium or in the synthetic H4 medium, the wild-type Lac$^-$ MC4100 strain transformed with pSH241 produced high levels of β-galactosidase (Table 1). However, the same plasmid introduced into HP4110, an *himA* derivative of MC4100, showed drastically reduced levels of β-galactosidase. The higher β-galactosidase activity seen with H4 medium than with TYE medium is reminiscent of earlier findings (16) that some components present in the complex TYE medium prevent full induction of TdcB.

The decreased expression of β-galactosidase from the *tdc* promoter in the *himA* strain was directly corroborated by assaying threonine dehydratase activities from the chromosomally encoded *tdcB* gene: in TYE medium, MC4100 (*himA*$^+$) had 762 U of dehydratase per mg of protein, whereas strain HP4110 (*himA*) showed 106 U/mg of protein, or 14% of the wild-type enzyme activity. We interpret these data to mean that a functional IHF protein is required for optimal expression of the *tdc* genes.

As mentioned above, the nucleotide sequence of the *tdc* promoter region (Fig. 1) contains the sequence 5' CTT TAACTTGTTGATA 3' between nt −118 and −103. This sequence has significant homology (shown by bold letters) with the consensus binding sequence 5' WATCAAN NNNTTR 3' (where W = A or T, R = A or G, and N = any base) for IHF, originally proposed for the λ recombinant site *attP* (4) and further refined by footprinting and mutational analyses (9, 19). It seemed plausible that IHF could occupy this site on the *tdc* promoter to enhance transcription. To test this notion, we introduced two mutations in this region by (i) substituting three bases (A→G, T→C, and T→C at positions −113, −107, and −104, respectively) to yield the plasmid pYW75 and (ii) deleting a 17-bp sequence extending from −118 to −102 (Fig. 4); two additional substitutions in the deletion mutant, designated pYW76, generated a new restriction site to facilitate screening. Assay of β-galactosidase activities in strain MC4100 (*himA*$^+$) transformed with the mutant plasmid pYW75 and incubated anaerobically in TYE medium and H4 medium showed about 10% of the enzyme activity seen with MC4100(pSH241), which harbors the wild-type IHF sequence (Table 1). The low levels of β-galactosidase in the IHF-negative strain with the wild-type plasmid IHF sequence [HP4110(pSH241)] and in the IHF-positive host with the mutated plasmid IHF sequence [MC4100(pYW75)] clearly suggest that both a functional IHF protein and the IHF-binding site on the wild-type *tdc* promoter, recognized from its consensus sequence, are necessary for *tdc* operon expression in vivo.

Strain MC4100 (*himA*$^+$) transformed with the deletion mutant plasmid pYW76 showed, unexpectedly, a somewhat higher β-galactosidase activity (approximately 25% of the maximally expressed level) than the 10% value found with MC4100(pYW75) (Table 1). Strain HP4110 (*himA*) transformed with pYW76 also showed higher activity than did HP4110(pWY75). These results were consistently reproducible in many assays, and a plausible explanation of this finding is given in Discussion.

**Effect of *tdcR* on IHF-mediated *tdc* expression.** We previously reported (30) that the *tdcR* gene product acts as a *trans*-acting positive activator for the *tdc* operon. The codon preference statistics of *tdcR* and minicell expression of its product suggested that the *tdcR* gene is weakly expressed. Further, deletion analysis revealed that a DNA segment extending from the *tdcR-tdcA* promoter region and part of the *tdcR* coding sequence in pSH240 appears to contain a *cis*-acting site necessary for TdcR action (30). The following experiments were designed to decide whether IHF indirectly stimulated *tdcB-lacZ* expression by increasing synthesis of TdcR and whether the *tdcR* gene product had any influence

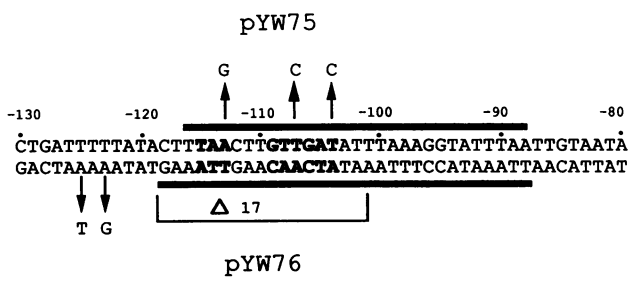


FIG. 4. Nucleotide sequence surrounding the IHF-binding site, showing the base substitutions and the 17-bp deleted region. The overlined and underlined areas represent sequences of noncoding and coding strands, respectively, protected by IHF against DNase I digestion (taken from the gels shown in Fig. 6).

TABLE 2. Effect of TdcR on TdcB-LacZ expression

| Expt | Strain and plasmid | Relevant genotype | Plasmid IHF sequence | LacZ sp act (%)[a] |
|---|---|---|---|---|
| I | MC4100(pSH241) | *himA*$^+$ *tdcR*$^+$/*tdcR*$^+$ | Wild type | 5,792 (100) |
| | MC4100(pYW75) | *himA*$^+$ *tdcR*$^+$/*tdcR*$^+$ | Substitution | 658 (11) |
| | MC4100(pYW76) | *himA*$^+$ *tdcR*$^+$/*tdcR*$^+$ | Deletion | 1,600 (27) |
| II | MC4100(pSH240) | *himA*$^+$ *tdcR*$^+$/*tdcR* | Wild type | 1,041 (18) |
| | MC4100(pYW752) | *himA*$^+$ *tdcR*$^+$/*tdcR* | Substitution | 146 (3) |
| | MC4100(pYW764) | *himA*$^+$ *tdcR*$^+$/*tdcR* | Deletion | 147 (3) |
| III | TH274(pSH240) | *himA*$^+$ *tdcR*/*tdcR* | Wild type | 304 (5) |
| | TH274(pYW752) | *himA*$^+$ *tdcR*/*tdcR* | Substitution | 205 (4) |
| | TH274(pYW764) | *himA*$^+$ *tdcR*/*tdcR* | Deletion | 175 (3) |
| IV | HP4110(pSH240) | *himA tdcR*$^+$/*tdcR* | Wild type | 185 (3) |
| | HP4110(pYW752) | *himA tdcR*$^+$/*tdcR* | Substitution | 169 (3) |
| | HP4110(pYW764) | *himA tdcR*$^+$/*tdcR* | Deletion | 172 (3) |
| V | MC4100(pMC1403) | *himA*$^+$ *tdcR*$^+$/vector | | 100 (2) |
| | TH274(pMC1403) | *himA*$^+$ *tdcR*/vector | | 123 (2) |
| | HP4110(pMC1403) | *himA tdcR*$^+$/vector | | 126 (2) |

[a] Conditions are as described in footnote *a* of Table 1, except that all the cells were incubated in H4 medium (16).

on IHF-mediated transcriptional activation of the *tdc* operon.

The plasmid pYW90 is a protein fusion plasmid in which the first 82 codons of *tdcR* are fused in frame to codon 7 of *lacZ*, and it contains the entire *tdcR-tdcA* promoter region. Assay of β-galactosidase activities of MC4100(pYW90) and HP4110(pYW90) incubated anaerobically in TYE medium revealed 13 and 16 U of enzyme, respectively, whereas the levels of β-galactosidase activity in the host strains transformed with the vector pMC1403 were less than 1 U of enzyme. As noted above, the low activity of β-galactosidase is most likely due to weak expression of the *tdcR* gene; nevertheless, the results clearly show that IHF has no effect on *tdcR'-'lacZ* expression. Thus, IHF appears to stimulate transcription of the *tdc* genes directly and not simply by increasing the level of TdcR in the cell.

To test the effect of TdcR on *tdcB'-'lacZ* expression, we monitored the β-galactosidase activities of strains MC4100 (*himA*$^+$ *tdcR*$^+$) and TH274 (*himA*$^+$ Δ*tdcR*) transformed with the *tdcB'-'lacZ* fusion plasmid pSH240 lacking *tdcR*. The results presented in Table 2 show that β-galactosidase activity in MC4100(pSH240), which contains a chromosomal copy of *tdcR*, was 18% of that found in the wild-type transformant, MC4100(pSH241) (cf. experiments I and II); enzyme activity was further reduced to 3% of the wild-type level when mutant IHF sequences replaced the wild-type IHF consensus region in the promoter of the fusion plasmids [Table 2, MC4100(pYW752) or MC4100(pYW764)]. In the complete absence of *tdcR*, as in TH274(pSH240), very little enzyme expression was seen even with a *himA*$^+$ host. In addition, 10% or less of the wild-type level of β-galactosidase activity was seen with HP4110 (*himA*) harboring chromosomal and/or plasmid-encoded *tdcR* [cf. HP4100(pSH241) in Table 1 and HP4110(pSH240) in experiment IV, Table 2]. We conclude from these data that both IHF and TdcR are essential for transcription of the *tdc* operon; lack of either protein drastically reduces the expression of the *tdc* genes. This conclusion is consistent with the observation reported previously (30) and reproduced here that an intermediate level of β-galactosidase expression was observed with MC4100(pSH240), presumably because one copy of the weakly expressed *tdcR* gene was not able to synthesize enough TdcR to maximize TdcB-LacZ production from the multicopy plasmid-encoded fusion. In a *himA*$^+$ strain, the concentration of IHF is not expected to be limiting because it is an abundantly expressed protein in *E. coli* (9). The results in Table 2 also confirm the notion that the putative IHF-binding sequence in the *tdc* promoter is necessary for IHF action, because mutations in the IHF consensus sequence decreased *tdc* operon expression even in the presence of IHF and TdcR.

**IHF binding to *tdc* promoter.** Having established the functional role of IHF in the transcriptional activation of the *tdc* operon, we employed two experimental methods to detect IHF binding to the *tdc* promoter and to localize its binding site. In the first method, appropriate DNA templates were incubated with cell extract from the *himA*$^+$ strain or with purified IHF to detect changes in mobility during electrophoresis through native polyacrylamide gel. As shown in Fig. 5, DNA fragment containing the wild-type promoter sequence (from nt −226 to +129) incubated with extracts of MC4100 (lanes 2 and 3) or purified IHF (lane 6) migrated slowly compared with what was seen with free DNA (lane 1). However, no shift in mobility was seen when the DNA fragment was incubated with extracts of strain HP4110 (*himA*) (lanes 4 and 5). Control experiments with a mutant template isolated from pYW75, which has three base substitutions in the highly conserved IHF consensus sequence (see Fig. 4), showed no retardation in DNA mobility when incubated with extract of MC4100 (Fig. 5, lane 8) or pure IHF (lane 9). Similar experiments performed with the DNA template isolated from the deletion plasmid pYW76 showed no mobility shift in the presence of pure IHF (data not shown), and a computer search of pYW76 DNA failed to detect a new IHF-binding site. These results clearly indicate binding of IHF to a region of the *tdc* promoter which harbors the IHF consensus sequence.

To directly determine the location of the IHF-binding site on the *tdc* promoter, the wild-type DNA template harboring the IHF consensus sequence was subjected to footprinting analysis as described by Craig and Nash (4). IHF protected the DNA segments from DNase I digestion between nt −118 and −88 of the coding strand (Fig. 6A, lane 3) and between −116 and −88 of the noncoding strand (Fig. 6B, lane 3). On the other hand, IHF did not protect either strand from DNase I digestion when the mutant IHF template was used for footprinting (Fig. 6A, lane 5 and 6B, lane 5). Thus, a

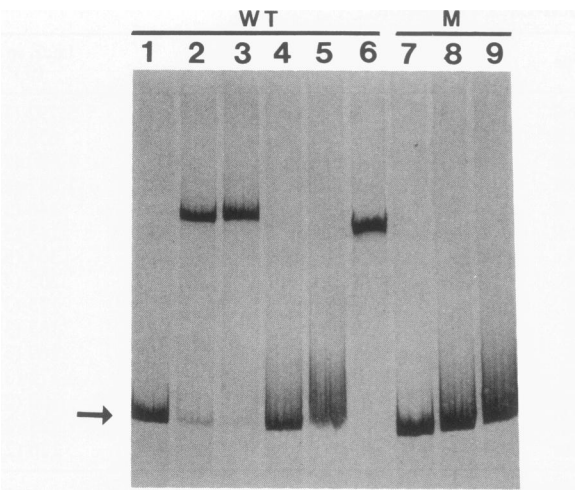


FIG. 5. Mobility shift of *tdc* promoter DNA due to binding of IHF. The 354-bp *DdeI-HpaI* labeled fragments from pSH241 (WT) and pYW75 (M) were incubated with various extracts or purified IHF and then electrophoresed on a 4% native polyacrylamide gel as described in Materials and Methods. The mobilities of free DNA (marked by an arrow) and DNA-IHF complexes were visualized by autoradiography. Lanes 1 to 6, wild-type DNA from pSH241; lanes 7 to 9, mutant DNA from pYW75. Lanes 1 and 7, free DNA; lanes 2 and 3, 10 and 20 μg of protein, respectively, from cell extract of MC4100 (*himA*$^+$); lanes 4 and 5, 10 and 20 μg of protein, respectively, from cell extract of HP4110 (*himA*); lane 6, 20 ng of purified IHF; lane 8, 20 μg of protein from MC4100 extract; lane 9, 20 ng of purified IHF.

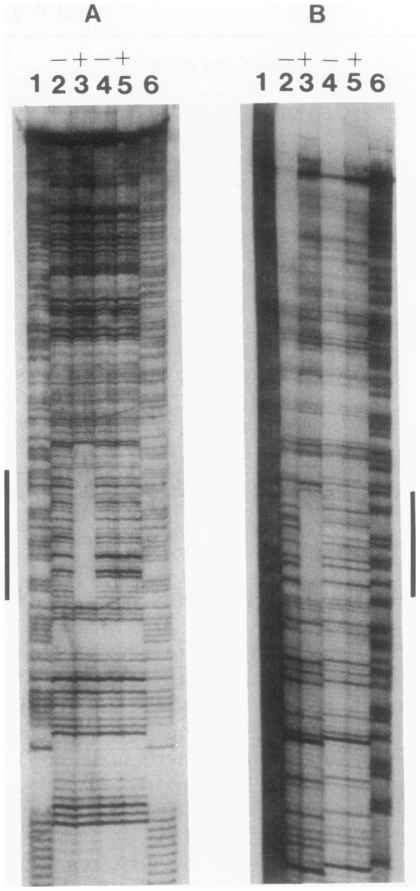


FIG. 6. Protection against DNase I digestion of the *tdc* promoter DNA fragment by purified IHF. Samples of uniquely end-labeled wild-type and mutant DNA templates were incubated with or without 20 ng of purified IHF and were then treated with DNase I as described in Materials and Methods. (A) Coding strands from wild-type promoter (lanes 2 and 3) and from mutant promoter (lanes 4 and 5); (B) noncoding strands from wild-type promoter (lanes 2 and 3) and from mutant promoter (lanes 4 and 5). Plus and minus signs represent presence and absence, respectively, of IHF before DNase I treatment. In both panels, lanes 1 and 6 are A > C sequencing ladders of the corresponding strands. The regions protected by IHF are marked by vertical bars.

31-bp region of the *tdc* promoter DNA which encompasses the consensus IHF-binding site appears to bind IHF in vitro.

## DISCUSSION

Transcriptional regulation of gene expression is a major control mechanism in both procaryotes and eucaryotes. Recent studies from this laboratory identified three separate proteins, namely, TdcA (26), TdcR (30), and CAP (6), as positive transcriptional activators of the *tdc* operon, and the experiments summarized here implicate IHF as an additional regulatory component necessary for *tdc* operon expression. Both CAP and IHF are generally considered to be global regulators of gene expression because they influence a wide variety of genes and cellular processes. On the other hand, TdcA and TdcR are specific for the *tdc* genes. Thus, the *E. coli tdc* operon, which is implicated in anaerobic energy metabolism, appears to be a highly regulated system.

How does a *trans*-acting activator protein facilitate transcription? Most procaryotic transcription-regulatory proteins examined thus far bind to their specific DNA sites within the target promoter. In this report, we show that IHF binds to the *tdc* promoter approximately 100 nt upstream of the transcription start site. The nucleotide sequence (13, 31) and preliminary footprinting data (38) also revealed that there is a CAP-binding site near −45 and that both IHF and CAP can simultaneously bind to their respective DNA sequences. We reported earlier that TdcR and TdcA are distinct proteins with no detectable sequence similarities between them; however, these proteins have unique polypeptide segments (26, 30) that exhibit significant homologies to the helix-turn-helix motifs of typical DNA-binding proteins (7, 22). Furthermore, deletion analysis indicated that a *cis*-acting sequence in the *tdcR-tdcA* promoter region is required for TdcR action (30). These findings suggest that both TdcR and TdcA could potentially function as DNA-binding proteins. It is tempting, therefore, to conclude that all four positive activators identified thus far—namely, TdcR, TdcA, CAP, and IHF—may facilitate *tdc* transcription by interacting with the *tdc* promoter. It is noteworthy in this context that the results presented in Table 2 suggest that IHF and TdcR can simultaneously occupy their respective DNA sites because both proteins are required for maximal *tdc* expression, and at a limited TdcR concentration supplied in *trans* by a single chromosomal copy of *tdcR*, the expression of β-galactosidase from the *tdcB'-'lacZ* fusion in an IHF-positive host was partially restored.

The 31-bp IHF-binding region in the *tdc* promoter includes a core sequence, 5′ CTTTAACTTGTTG 3′, which is different from the deduced consensus sequence of Friedman (9) at three positions (shown in bold letters in Fig. 7); the *tdcP* IHF site also deviates from the core IHF-binding sequence

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Consensus | W | A | T | C | A | A | N | N | N | N | T | T | R | |
| *tdcP* | **C** | **T** | T | **T** | A | A | C | T | T | G | T | T | G | |
| λ H′(I), H′(II) | T | A | T | C | A | A | T | T | T | G | T | T | G | |
| | 33 | | 35 | | 37 | | 39 | | 41 | | 43 | | 45 | |

FIG. 7. Core IHF-binding sequence in the *tdc* promoter (*tdcP*) and λ H′ recognition site. The consensus IHF sequence of Friedman (9) is shown at top. The numbers at bottom refer to the assigned base numbers at the λ H′ region (19). N, any base; W, A or T; R, G or A. Boldface letters are explained in Discussion.

in the λ H′ recognition site at the same three positions (bases 33, 34, and 36) as well as at position 39. These differences might indicate that IHF binds *tdcP* DNA weakly, yet the results of gel mobility shift and footprinting experiments with $\sim 10^{-8}$ M IHF, shown in Fig. 5 and 6, indicate strong *tdcP* DNA-IHF interaction. A careful examination of the mutational data of Lee et al. (19) on the bacteriophage P22-based challenge phage system, undertaken to study the binding of IHF to the λ H′ site, revealed that a T→C substitution at position 33 had a weak to moderate effect on disruption of IHF binding. Also, a C→T base change at position 36 severely disrupted IHF binding to a phage H′(I) derivative, but it did so less severely in phage H′(II). These authors did not find any mutations at positions 34 and 39 to evaluate the importance of these bases in IHF binding. These considerations imply that some other features in the *tdc* promoter, such as DNA topology and/or bases outside the core region, may also contribute to the tight binding of IHF.

An unexpected observation emerged during β-galactosidase assays of MC4100 (*himA*$^+$) and HP4110 (*himA*) strains transformed with the *tdcB′-′lacZ* plasmid (pYW76), which harbors a deletion of the entire consensus IHF-binding sequence. In repeated assays, MC4100(pYW76) exhibited, on average, approximately 25% of the maximally expressed level of β-galactosidase activity, whereas only about 10% of maximal enzyme activity was found with MC4100(pYW75), which contains three base substitutions in the wild-type IHF sequence. Similarly, HP4110(pYW76) showed, on average, about fourfold higher basal enzyme activity than did HP4110(pYW75) (Table 1). How do we account for the higher level of *tdc* transcription in the IHF sequence deletion mutant? Two pieces of information described below might explain this result. The *tdcR-tdcA* promoter DNA is highly A-T rich and exhibits the characteristics of bent DNA (32). Both CAP (37) and IHF (25) are also known to induce DNA bending near their respective target sites. It has been proposed that the function of IHF may just be to bend DNA to create an ordered structure to facilitate integrative recombination of λ phage (12), and some current models of transcriptional regulation of procaryotic genes by *trans*-acting regulatory proteins invoke DNA looping and/or DNA bending (1, 17, 39). It is plausible that deletion of the 17-bp DNA sequence (less than two complete helical turns) in the IHF-binding site of the *tdc* promoter might create a new structure in the promoter DNA that could partially mimic the optimum promoter configuration induced by IHF binding to wild-type DNA. If true, the deletion mutant is expected to exhibit partial enzyme activity in the absence of IHF binding. This notion is supported by the finding that in the IHF-negative strain, a significantly high basal level of enzyme activity was expressed from the deletion plasmid pYW76 (Table 1). As already mentioned, a computer search of pYW76 DNA failed to detect a putative IHF-binding sequence, and gel retardation analysis showed no evidence of IHF binding to pYW76. Interestingly, in a separate experiment we found that TH274(pYW76), which lacks the chromosomal *tdcR* gene, had about twofold higher basal β-galactosidase activity (531 U) than was seen with TH274(pYW75) (270 U), whereas enzyme activities from both pYW752 and pYW764, which lack plasmid *tdcR*, were low in *tdcR*$^+$ and *tdcR* hosts (Table 2; cf. experiments II, III, and IV). These results indicate that a high level of TdcR, supplied by the plasmid-borne *tdcR* gene, is needed for higher basal enzyme activity from the deletion plasmid pYW76; a low level of TdcR expressed by the chromosomal copy of *tdcR* was not sufficient for TdcB-LacZ expression. It may be recalled that a high level of TdcR was also needed for β-galactosidase synthesis by the plasmid containing the wild-type IHF sequence (Table 2; cf. experiments I and II). Further investigation is under way to elucidate the structure-function relationships in the *tdc* promoter in terms of transcriptional activation by regulatory proteins.

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