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Characterization of *BNT2*, an intrinsically curved DNA of *Escherichia coli* O157:H7

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Abstract

The gene regulation by intrinsically curved DNA is one way for bacterial sensing of and response to environmental changes. Previously, we showed that the genetic element *BNT2* upstream of the *ecf* (*eae*-positive conserved fragment) operon in the *Escherichia coli* O157:H7 virulence plasmid (pO157) has characteristics typical of intrinsically curved DNA, including the presence of multi-homopolymeric adenine:thymine tracts (AT tracts) and electrophoretic anomaly at 4°C. Here we report that a local intrinsic curvature induced by the two phased AT tracts within the unusual promoter sequence of *BNT2* played a major role for its temperature-dependent promoter activity. The base substitution of the AT tract in the spacer DNA between the -35 and the unusual -10 regions of the *BNT2* promoter with non-AT tract sequence reduced intrinsic curvature slightly at 4°C, but greatly affected its transcriptional activity. This implies that such a local intrinsic curvature within the unusual promoter of *BNT2* is important for thermoregulation of the *ecf* operon.

Keywords

intrinsic curvature; *BNT2*; AT tract; thermoregulation; *ecf* operon; *Escherichia coli* O157:H7

Introduction

Escherichia coli O157:H7 is an important food-borne bacterial pathogen that causes hemorrhagic colitis and hemolytic uremic syndrome in humans and animals [1]. Interestingly, the bacterium is well known to be hardy in diverse *in vivo* and *ex vivo* environments. For example, *E. coli* O157:H7 can survive passage through the extremely acidic gastric stomach (pH<1.5) [2;3], enhance virulence expression at the gastrointestinal milieu (i.e. induced

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expression of the genes in the LEE pathogenicity island) [4;5], and persist in various environmental sources such as soil [6], raw manure [7], and farm water [8]. Therefore, it is likely that coordinated regulation of a set of *E. coli* O157:H7 genes might be required for this environmental flexibility although the underlying mechanisms are not clearly understood.

One of the environmental sensing and response mechanisms in cells is the gene regulation by intrinsically curved DNA, previously defined as DNA segments with a curved trajectory in its helix axis [9]. Intact nucleotide sequences such as short runs of homopolymeric adenine:thymine residues (AT tracts) is known to induce the intrinsic curvature of DNA especially when inserted in phase with the helical periodicity of 10 to 11-bp interval [9;10]. Such changes in intrinsic DNA topology within or upstream of the promoter sequence may promote or inhibit binding of RNA polymerase (RNAP) and/or other DNA binding proteins (i.e. a nucleoid-associated protein H-NS, stationary sigma factor σ^S , or regulatory proteins HU, IHF, and FIS *etc.*) to the curved region of DNA [11;12], which in turns modulates gene expression at the level of transcription. Interestingly, some environmental factors including temperature, pH, osmolarity, ionic strength such as $[Mg^{2+}]$, and anaerobiosis can affect gene expression by intrinsically curved DNA [13;14;15], suggesting that intrinsically curved DNA coordinates environmental signals with gene expression.

Some virulence-associated genes in bacterial pathogens are known to be controlled by intrinsic DNA curvature in response to certain environmental conditions. Such examples include transcriptional activation of type IV bundle forming pili in enteropathogenic *E. coli* [16;17;18] and VirF in *Shigella flexneri* and *Yersinia enterocolitica* [19;20] during a temperature shift from 24 to 37°C. Moreover, a recent study reported that intrinsic curvature signals of DNA are highly conserved in putative regulatory regions of bacterial genomes, supporting their function as a global regulatory mechanism for prokaryotic genes [21].

Previously, we isolated *BNT2* intrinsically curved DNA from the *E. coli* O157:H7 virulence plasmid (pO157) [22]. *BNT2* has characteristics typical of intrinsically curved DNA such as electrophoretic anomaly at 4°C, six partially phased AT tracts, and the temperature-dependent transcription of the pO157-encoded *ecf* (*cae*-positive conserved fragments, *ecf1* to 4) operon [22] whose gene products are involved in the structural modification of the outer membrane lipopolysaccharide (LPS) [22;23;24;25]. For example, *ecf1* and *ecf2* encodes a putative polysaccharide deacetylase and a functional LPS α -1, 7-N-acetylglucosamine transferase, respectively and both are unique to pO157 [26]. *ecf3* shows similarity to a putative outer membrane protein in *E. coli* K1 associated with bacterial invasion [27]. *ecf4* encodes the second copy of a lipid A myristoyl transferase [22;25]. The double mutant carrying deletions in the *ecf4* and its chromosomal copy *lpxM* of *E. coli* O157:H7 had an altered lipid A structure based on growth temperature and membrane fatty acid composition, and showed decreased persistence in the bovine gastrointestinal tracts [22;24;26].

Previous biochemical characterization of *BNT2* identified a functional promoter sequence with an unusual -10 (AAAAAT) element. Interestingly, two AT tracts are positioned in phase between this unusual -10 element and the spacer DNA in the promoter sequence of *BNT2*, suggesting the possibility of an AT tract-induced intrinsic curvature at this defined region [22]. In this study, therefore, we hypothesized that the AT tract-induced intrinsic curvature between the spacer DNA and the unusual -10 element of *BNT2* contributes to the promoter activity of *BNT2* that depends on temperature. To test this hypothesis, the AT tract in the spacer DNA was replaced with non-AT tract sequence by site-directed mutagenesis and its biological significance was examined by measuring both intrinsic DNA curvature and the promoter activity at the different temperatures.

Materials and Methods

Bacterial strains, plasmids, and *BNT2* nucleotide sequence

Bacterial strains and plasmids used in this study are listed in Table 1. All bacterial strains were grown and maintained in Luria-Bertani (LB) media (MP Biomedicals) with or without 1.5% (w/v) agar. If necessary, the antibiotics were used at the following concentrations: ampicillin (Ap), 100 µg/ml and kanamycin (Km), 50 µg/ml.

The DNA nucleotide sequence of *BNT2* was obtained from the GenBank database (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide>) available at National Center for Biotechnology Information (NCBI) with the accession number AY330225.

General procedures

General molecular techniques including polymerase chain reaction (PCR), gene cloning, plasmid purification, and transformation were carried out as previously described [28]. All the enzyme reactions were followed by the manufacturers' recommendation and DNA sequencing analyses were done by MacroGen Inc. (Seoul, Korea), which was commercially available.

Site-directed mutagenesis by inverse PCR

A 1,041-bp *BNT2* fragment from pSKBNT2 [22] was cloned into the RBC TA cloning vector (Real Biotech Corporation). The resultant plasmid was confirmed by DNA nucleotide sequencing and designated pRBC/*BNT2* (Table 1).

To disrupt a local intrinsic curvature within the unusual promoter sequence of *BNT2*, the AT tract (AAAAAT) in the spacer DNA of *BNT2* (near +960-bp position) was substituted with non-AT tract sequences (TCG) by introducing the *Cla*I restriction site, yielding *AT24M*. Briefly, the *Cla*I site-incorporated primers, J11 (5'-TGATATCGATATTAATAATCCCTGTTGTCGG-3') and J12 (5'-ATATCGATATCAGTTAATCAACCAGAACT-3'), were used for inverse PCR with the template plasmid pRBC/*BNT2* (Table 1). The amplified PCR products were purified from the agarose gel using HiYield Gel/PCR DNA extraction kit (Real Biotech Corporation), digested with *Cla*I (New England Biolabs), and self-ligated with T4 ligase (New England Biolabs). The resultant plasmid was confirmed by DNA nucleotide sequencing and designated pRBC/*AT24M* (Table 1).

The 134-bp promoter regions at the 3'-end of *BNT2* or *AT24M* (corresponding to the nucleotide positions from +907 to +1,041-bp) were obtained by PCR using the template plasmids, pRBC/*BNT2* or pRBC/*AT24M*, with the primers J86 (5'-GGGGAATTTCGAGCATATGCGTTAAGGGAA-3') and M13R (5'-GGAAACAGCTATGACCATG-3') and subcloned into the RBC TA cloning vector. The resultant constructs were confirmed by DNA sequencing and designated pRBC/*BNT2*-134 or pRBC/*AT24M*-134, respectively (Table 1).

In silico prediction of intrinsic DNA curvature

The bend.it server (http://hydra.icgeb.trieste.it/dna/bend_it.html) was used to calculate the intrinsic curvature of *BNT2* or *AT24M* DNA molecules as predicted from their DNA nucleotide sequences. This prediction was based on values tabulated for di- or tri-nucleotides and the intrinsic curvature was calculated using standard algorithms [29] with a window size of 31-bp. For visualizing the 3-dimensional structures of *BNT2* and *AT24M*, the model.it server (http://hydra.icgeb.trieste.it/dna/model_it.html) was used as followed by the server's direction.

Experimental detection of intrinsic DNA curvature

To analyze the electrophoretic gel retardation of intrinsically curved DNA at 4°C, the gel-purified DNA fragments were subject to one-dimensional (1-D) polyacrylamide gel electrophoresis (PAGE) at 60 or 4°C on 5% non-denatured polyacrylamide gels. Based on the molecular marker, the expected sizes of migrated DNA fragments at both temperatures were calculated and compared using Microsoft Excel program (version 2003, Microsoft Corporation).

For more sensitive detection of intrinsic DNA curvature, two-dimensional (2-D) PAGE was used as previously described [20]. Briefly, one microgram of pRBC/BNT2-134 or pRBC/AT24M-134 was digested completely with *HindIII* at 37°C, mixed with 1 ug of 100-bp DNA ladder (Invitrogen), and separated on 10 or 15% non-denaturing polyacrylamide gels. After the first dimensional electrophoresis at 60°C, the glass-gel sandwich was rotated and transferred to pre-chilled 1×TBE buffer (45 mM Tris-borate, 1mM EDTA, pH8.4) and subject to the second dimensional electrophoresis at 4°C. The gel was stained with ethidium bromide (EtBr) and visualized using the Core-Bio iMAX image analysis system (Core-Bio Inc.).

Determination of transcriptional activity

To avoid possible biases from heterogeneous strains or plasmid copy-number effects, a single-copy *BNT2::lacZ* transcriptional fusion system was constructed on the chromosome of *E. coli* O157:H7 ATCC 43894 by allelic exchange as previously described [30]. Briefly, *BNT2* was ligated into *EcoRI* and *BamHI* sites of pRS551, a multi-copy *lacZ* transcriptional fusion plasmid [31], resulting in pRSBNT2. The 6.0-kb *ScaI-SacI* fragment of pRSBNT2 was ligated with 8.7-kb *EcoRV-SacI* fragment of the conjugative suicide vector, pJRLacZins [32]. After transformation into *E. coli* S17-1 λ pir, the resulting construct was transferred to the wildtype *E. coli* O157:H7 strain by conjugation and recombined into intact *lacZ* gene on the *E. coli* chromosome. The *E. coli* O157-positive, Ap-resistant (Ap^R) and Km^R exconjugants were counter-selected under presence of 6% sucrose and finally, the Km^R/Ap^S colonies were confirmed by PCR.

β-galactosidase activity

Transcriptional fusion strains were grown in LB containing appropriate antibiotics with or without 0.4% glucose at various temperatures such as 10, 15, 24, 37, and 45°C with shaking at 150 rpm to an optical density at 600 nm (OD₆₀₀) of 0.5. The β-galactosidase activity was measured with 0.2% sodium dodecyl sulfate/chloroform permeabilized cells as previously described [33]. At least, three independent experiments were performed and the data were analyzed statistically by the student t-test using Microcal Origin™ (version 8.0, Microcal Software, Inc.).

Construction of a multi-copy AT24M::lacZ transcriptional fusion

The transcriptional fusion vector pSP417 carrying a promoterless *lacZ* gene [34] was used to analyze the transcriptional activity of *BNT2* or *AT24M*. The 1,041-bp *BNT2* or *AT24M* fragments were restricted by both *EcoRI* and *BamHI* and ligated into the *EcoRI* and *BamHI* sites of pSP417, resulting in pSPBNT2 and pSPAT24M (Table 1). The resultant plasmids were transformed into *E. coli* strains and screened for appropriate antibiotic resistance.

Results and Discussion

Temperature dependency of the transcriptional activity of *BNT2* intrinsically curved DNA

A typical characteristic of intrinsically curved DNA is electrophoretic gel retardation at low temperatures, which is due to temperature-dependent changes in DNA topology. Consistent

with our previous observation [22], *BNT2* intrinsically curved DNA showed slowed migration at 4°C compared to 60°C (Fig. 1A). Indeed, the 1.0-kb sized fragments of *BNT2* at 60°C migrated like the 1.6-kb sized fragments at 4°C (Fig. 1A). Since *BNT2* is known to have a functional promoter whose activity relies on growth temperatures [22], temperature dependency of the *BNT2*-driven transcriptional activity was investigated at various temperatures ranging from 10 to 45°C (Fig. 1B). Our results demonstrated that *BNT2*-driven transcription was significantly high at low temperatures such as 10, 15, and 24°C with a peak activity at 24°C, compared to at high temperatures 37 and 45 °C ($p < 0.05$, Fig. 1B). Indeed, approximately 3.1-fold higher activity in transcription was observed at low temperatures (10, 15, and 24°C) compared to at high temperatures (37 and 45 °C), regardless of the presence of glucose (Fig. 1B). These results imply that DNA relaxation at high temperatures above 37°C may affect the *BNT2*-driven transcriptional activity which depends on temperature. Supporting this notion, a previous study demonstrated that the intrinsic DNA curvature in the *Y. enterocolitica* virulence plasmid (pYV) was relaxed at between 30 and 37°C [20].

Site-directed mutagenesis of the AT-tract in the spacer DNA of the unusual promoter sequence of *BNT2*

The previous genetic mapping of *BNT2* has identified the six partially phased AT tracts located upstream of the transcriptional start site (a guanine residue in Fig. 2) of the *E. coli* O157:H7 pO157 *ecf* operon (Fig. 2). Among those six AT tracts, two AT tracts were positioned in phase with a 10-bp periodicity and localized within the functional, but unusual promoter sequence of *BNT2* (Fig. 2). To examine if a possible intrinsic curvature induced by those two phased AT tracts within the unusual promoter sequence of *BNT2* contributes to the *BNT2*-driven promoter activity responding to temperature, we targeted and replaced the AT tract in the spacer DNA with non-AT tract sequence by site-directed mutagenesis (Fig. 2). Defined mutation was confirmed by DNA nucleotide sequencing (data not shown). The resultant DNA fragment, *AT24M*, had intact -35 and -10 elements of *BNT2*, but contained non-AT tract sequence in the spacer DNA region unlike in *BNT2* (Fig. 2).

In silico modeling of *BNT2* and *AT24M*

To investigate whether or not the base substitution of the AT tract in the spacer DNA with non-AT tract (Fig. 2) could disrupt a possible local intrinsic curvature, the nucleotide sequences from *BNT2* or *AT24M* were predicted for the presence of intrinsic curvature (see Materials and Methods). As expected, the results revealed that a predicted intrinsic curvature in the 134-bp promoter region at the 3'-end of *BNT2* became reduced at the corresponding region at *AT24M* where the defined base substitution was introduced (Fig. 3A). Consistently, *in silico* modeling of both *BNT2* and *AT24M* indicated that the 134-bp promoter region of *AT24M* was relaxed by approximately 16 degrees compared to the corresponding region of *BNT2* (Fig. 3B).

Biological significance of the AT tract-induced intrinsic curvature within the unusual promoter sequence of *BNT2*

The predicted intrinsic DNA curvature present in *BNT2* and *AT24M*, especially within their promoter regions, was confirmed by 2-D PAGE (Fig. 4A). The *Hind*III-restricted DNA fragments containing the 134-bp promoter regions from the 3'-end of *BNT2* or *AT24M* were purified from pRBC/*BNT2*-134 or pRBC/*AT24M*-134, respectively, and analyzed on both 10 and 15% non-denatured polyacrylamide gels (Fig. 4A). Consistent with our *in silico* modeling, the restricted fragments of *BNT2* showed a slightly retarded migration at 4°C compared to those of *AT24M* on both 10 and 15% non-denatured gels (Fig. 4A).

Can such a subtle change in DNA topology of *BNT2* affect the *BNT2*-driven promoter activity responding to temperature? To address this question, the transcriptional activity of *BNT2* and *AT24M* was examined at 37 and 24°C. As shown in Fig. 4B, surprisingly, the significant

changes in transcription were observed between *BNT2* and *AT24M* at both temperatures ($p < 0.05$, Fig. 4B). At 24°C, the *BNT2*-driven transcription was about 4.6 folds higher than that at 37°C, while the *AT24M*-driven transcription was about 2.1 folds higher than that at 37°C (Fig. 4B). Although a simple base substitution of the AT tract in the spacer DNA did not fully turned off the promoter activity of *BNT2*, it significantly affected the *BNT2*-driven transcription as well as its temperature dependency. Taken together, these results imply that a local intrinsic curvature induced by the two phased AT-tracts within the unusual promoter sequence of *BNT2* are important for the temperature-dependent transcription of the pO157-encoded *ecf* operon.

In conclusion, bacterial coordination of environmental signals with gene expression is most important for their survival and persistence in various environmental conditions as well as contributes to an established infection *in vivo*. Not surprisingly, a recent study has reported that DNA curvature signals are conserved in regulatory regions of a cluster of prokaryotic genes such as proteins HU and IHF, DNA gyrase subunits A and B, FIS protein-coding genes, transposases, cell division-related genes, and flagellum genes [21]. Our data suggest that the gene regulation through intrinsic DNA curvature especially within or near the promoter sequences seems to be very sensitive to environmental changes because only a subtle change in intrinsic DNA topology within the unusual promoter sequence of *BNT2* greatly reduced its transcription. It would be interested to elucidate the mechanisms behind this regulation as well as to identify other *in vivo* or *ex vivo* environmental signals which can modulate the *BNT2*-driven promoter activity.

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Fig. 1A.

Fig. 1B

Fig. 1. Electrophoretic anomaly of *BNT2* at 4°C

(A) One-dimensional polyacrylamide gel electrophoresis of *BNT2*. Each purified DNA was separated on 5 % polyacrylamide gel at 60°C or 4°C to examine intrinsic curvature. (B) Temperature dependency of the *BNT2*-driven transcription. The strain (YH2021) carrying a single-copy chromosomal *BNT2::lacZ* transcriptional fusion construct in ATCC 43894 was grown in LB with (filled bar) or without 0.4% glucose (open bar) at various temperatures indicated and β-galactosidase activity was measured. The enzyme activities are represented as the means ± standard deviation from at least three independent experiments.

Fig. 2. Site-directed mutagenesis of the AT tract in the spacer DNA of the unusual promoter sequence of *BNT2*

The schematic diagram of the *BNT2* containing region in pO157 is shown. The nucleotide base position in pO157 is indicated at the top left and right. The black boxes indicate the AT tracts and the arrow shows the transcriptional start site (+1 and the asterisk). The capitalized letter P in a circle represents the functional, but unusual promoter sequence of *BNT2*. The AT tract in the spacer DNA region between the -35 element and the unusual -10 element of *BNT2* was replaced with non-AT tract sequences by PCR mutagenesis (see Materials and Methods). The resultant mutation was directly confirmed by DNA nucleotide sequencing.

Fig. 3A

Fig. 3B

Fig. 3. Prediction of intrinsic DNA curvature (A) and *in silico* modeling (B) of *BNT2* and *AT24M*
 Individual nucleotide sequence was analyzed for intrinsic curvature using the bend.it server. The 134-bp promoter regions containing the two phased AT tracts from *BNT2* and *AT24M* were reconstituted by the model.it server. The arrow indicates the defined spacer DNA regions between the -35 element and the unusual -10 element of *BNT2* or *AT24M* (panel A). In the panel B, changes in intrinsic DNA curvature were compared by measuring the angles produced by the two artificial lines (dotted lines).

Fig. 4A

Fig. 4B

Fig. 4.

Involvement of a local intrinsic curvature within the unusual promoter sequence of *BNT2* in its temperature-dependent transcription. (A) The *Hind*III-restricted fragments containing the 134-bp *BNT2* or *AT24M* promoter regions were subjected to 2-D PAGE as indicated with 1.0 μg of the 100-bp ladder DNA marker (Gibco BRL). The 2-D gels were visualized with UV light after EtBr staining. The arrow indicates the DNA fragments containing the 134-bp *BNT2* or *AT24M* promoter regions. The dash lines showed the migration status of the 200-bp DNA marker band on the 2-D gels and used to compare to the migration status of the DNA fragments containing the 134-bp *BNT2* or *AT24M* promoter regions. (B) Temperature-dependent transcriptional activity of *BNT2* and *AT24M*. The multi-copy transcriptional fusion

plasmids of *BNT2* (pSPBNT2) or *AT24M* (pSPAT24M) were constructed on a promoterless operon fusion vector (pSP471) and transformed into *E. coli* DH5 α . The *lacZ* expression was measured as β -galactosidase activity after grown in LB at 37 or 24°C. The enzyme activities are represented as the means $\pm$ standard deviation from at least three independent experiments.

Table 1
Bacterial strains and plasmids in this study

Strain / plasmid	Description	Reference
<i>E. coli</i> strains:		
DH5a	A general cloning host	RBC ^a
ATCC 43894	<i>E. coli</i> O157:H7 (a human clinical isolate, <i>stx</i> ₁ ⁺ / <i>stx</i> ₂ ⁺)	ATCC ^b
YH2021	ATCC 43894 with a single copy <i>BNT2::lacZ</i>	This study
Plasmids:		
pSKBNT2	1,041-bp <i>BNT2</i> fragments in pBluescript SK+	[22]
pRBC/BNT2	<i>BNT2</i> fragments in RBC TA cloning vector	This study
pRBC/AT24M	<i>AT24M</i> in RBC TA cloning vector	This study
pRBC/BNT2-134	a 134-bp region of <i>BNT2</i> in RBC TA cloning vector	This study
pRBC/AT24M-134	a 134-bp region of <i>AT24M</i> in RBC TA cloning vector	This study
pRS551	a <i>lacZYA'</i> operon fusion vector (pBR322 origin, Ap ^R Km ^R)	[31]
pRSBNT2	<i>BNT2</i> in pRS551 (Ap ^R Km ^R)	[22]
pSP417	A <i>lacZYA'</i> operon fusion vector (pBR322 origin, Ap ^R)	[34]
pSPBNT2	<i>BNT2</i> in pSP417 (Ap ^R)	[22]
pSPAT24M	<i>AT24M</i> in pSP417 (Ap ^R)	This study

^aReal Biotech Corporation, Taiwan

^bAmerican Type Culture Collection, Manassas, VA