Adaption of COBRA Method for The Investigation of Nutrition-Based DNA Methylation Mechanism in Escherichia coli

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ABSTRACT: Epigenetics are changes that occur in the DNA sequence due to environmental conditions. In last few decades epigenetics has been one of the most important advances in biology and related fields. It is very important in that it offers a new approach to all relevant disciplines, especially biology, to explain how DNA sequencing in an epigenetic organism is affected by environmental conditions and phenotype changes that may result from this interaction. Although there are many environmental factors affecting organisms, starvation is one of the most important factors. In our study, epigenetic changes in Escherichia coli due to carbon starvation in the sprE (rssB) gene region were investigated by COBRA (Combined Bisulfite Restriction Analysis) technique, which is one of the quantitative methylation analyzes. As a result of the current results, it has been shown that the methylation status of the 16th CpG islet located in the sprE gene region does not play a role in the regulation of fasting metabolism in E. coli due to the restriction of the carbon level.

Keywords: Capillary gel electrophoresis, COBRA, DNA methylation, Escherichia coli, sprE gene

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INTRODUCTION

The term epigenetics was first introduced to explain how cell lines were shaped during the development of multicellular eukaryotes in 1942 by Conrad Waddington, (Handy et al., 2011; Tollefsbol, 2011; Yaykaşlı et al., 2012; Casadesús and Low, 2013; Gulluce et al., 2014; Jurkowski et al., 2015). It is widely known that epigenetic control of gene expression plays an important role in the processes of cell differentiation in vertebrates (Takahashi, 2013). At this point, most textbooks and study books on epigenetic gene regulation only concern eukaryotes. One reason for this is the great success of eukaryotic epigenetics and its effects on human diseases (Casadesús and Low, 2013).

Although there are many different types of classification, epigenetic mechanisms that regulate gene expression fall into two sub-categories, mainly indirect and direct role mechanisms. Among these, the mechanisms that directly take part; Acetylation, methylation, phosphorylation, S-nitrosylation, SUMOylation, ADP-ribosylation, proline isomerization, deimination and non-covalent histone modifications (Gulluce et al., 2014; Alaylar et al., 2019). All these epigenetic modifications are known to be altered by external and internal environmental factors and affect gene expression. Thus, epigenetics can provide a new research framework for environment-related etiologic factors, as well as embryonic development and aging are known to be affected by many environmental factors (Choi and Friso, 2010).

Nutrition is defined as one of the most important environmental factors known. Therefore epigenetics is extremely important as it can alter the gene expression and epigenetic events of nutrients and bioactive food components at transcriptional levels. Folate, vitamin B-12, methionine, choline and betaine may affect DNA methylation and histone methylation through alteration of one carbon metabolism (Choi and Friso, 2010; Takahashi, 2013).

It is known that DNA methylation is one of the most important nutrient-related epigenetic mechanisms. This phenomenon is a vital mechanism in normal embryonic development. The DNA methylation event, which changes a cytosine base in CpG dinucleotide residues with methyl groups, is catalyzed by Dnmt (DNA methyl-transferase enzymes) and modulates gene expression motifs by altering chromatin structures (Choi et al., 2009; Lillycrop and Burdge, 2015; Warzak et al., 2015).

Natural microbial environments often require adaptation to various stresses, such as food hunger, which can occur quickly. Escherichia coli responds to fasting stress by entering a state where it is metabolically less active, known as the fixed phase. As one of the proteins with the highest degree of expression regulation in E.coli, RpoS draws attention. After binding of the sprE protein to RpoS, RpoS accumulation in cells is thought to be affected and regulates the transcription of target genes (Carabetta et al. 2009).

Many techniques have been developed to show methylation events, which are largely responsible for the emergence of epigenetic arrangements in living things, and especially methods based on bisulfite reaction that allows rapid diagnosis of methylated cytosine (5-MeC) in DNA. Methylation-specific PCR (MSP), bisulfite sequencing and COBRA (combined bisulfite restriction analysis) methods are among the most well-known of these applications (Kosova et al., 2011). Among all these methods aimed at measuring the methylation status of DNA, applications involving the combination of PCR and Restriction enzymes have given a new perspective and momentum to existing epigenetic studies by providing quantitative results (Alaylar, 2014).

In this study was designed to quantitatively investigate the methylation motifs that may occur in the regulation of the sprE (rssB) gene region activated by nutritional starvation stress in Escherichia coli by COBRA method. Also in the current study, it is aimed to contribute to filling the theoretical gap related to epigenetic mechanisms existing in prokaryotic organisms.
MATERIALS AND METHODS

Chemicals
Sodium chloride (NaCl) was provided from Merck (Hohenbrunn, Germany). Chloroform-isooamyl alcohol were provided Fluka (Steinheim, Germany), phenol-chloroform-isooamyl alcohol 25:24:1 were provided Amresco. All other compounds such as; isopropanol, glycerol, ethylenediaminetetraacetic acid (EDTA), Tris-HCl, Direct Load™ wide-range DNA marker, CTAB, SDS, RNase, DMSO, Trizma base, Ethidium bromide and ethanol were purchased from Sigma-Aldrich (St. Louis, USA). The restriction enzymes for the COBRA analysis, TaqI and HinfI were obtained Thermo-Scientific (Waltham, MA USA). Wizard genomic DNA purification kit and Methyledge™ bisulfite conversion system were purchased from Promega (Madison, USA). Agarose was provided by Lonza. Expand high fidelity PCR system was acquired from Roche (Mannheim, Germany). Nutrient Broth and Nutrient Agar, Potato Dextrose Agar, bacteriological peptone and agarose acquired from Oxoid (Hampshire, England), LAB M (Lancashire, England) and Lonza (Rockland, USA) respectively.

Escherichia coli Strain
The bacterial strain Escherichia coli WP2uvrA (ATCC® Number: 49979) used in this study was commercially available from LGC Standards (Middlesex, U.K.).

Medium
Carbon Source Restricted Test Media is used to expose the test microorganism to carbon deficit. In preparation of this medium, 980 ml of distilled water is sterilized by autoclaving for 30 minutes at 121°C. After this process, the previously sterilized Vogel-Bonner (VB salts) medium E (50 ×) solution is added to the sterile water medium whose temperature is lowered to 45-65°C, and it is mixed well by gentle agitation in the sterile cabinet. This medium should be stored in sterile environments at room temperature (Mortelmans and Zeiger, 2000; Karadayı, 2010).

Preparation of Test Samples
Stock cultures were thawed at room temperature before working cultures were prepared. Then, 200 μl of stock culture was added to NB liquid medium, each having a volume of 40 ml. Prepared working cultures were allowed to incubate at 37°C at 110 rpm for 48 hours. After this time, one of the growing group cultures was transferred to a new NB medium and the other group was transferred to a carbon-restricted medium and these new cultures were left to the second 24-hour incubation period. DNA isolation, sodium bisulfite application, High Fidelity PCR, restriction enzyme cutting and imaging applications were performed with these cultures.

Genomic DNA Isolation
Genomic DNA was isolated from Escherichia coli. DNA isolation protocol was explained as follows.
Cultures were grown in NA media, and then two loops of bacteria are placed in eppendorf and shaken well after vortexing 1000 μl of STE buffer is added. The samples are centrifuged at 10000 rpm for 10 minutes and the supernatant is discarded. 1000 μl of STE (Sodium Chloride-Tris-EDTA) buffer is then added again and centrifuged at 10000 rpm for 10 minutes at 4°C. After the centrifugation process is completed, the supernatant is discarded and 500 μl of STE buffer is added to the pellet and mixed in every 15 minutes for a total of 30 minutes in the water bath previously set to 75°C. At the end of the period, 10% SDS is added to eppendorf and mixed. After this, 8 μl of Proteinase K is added and shaken again. After rinsing, it is suspended for 1 hour in a water bath set to 40°C. Add 1/5 of the total volume, 5M NaCl to the samples taken from the water bath. 10% by volume of CTAB
(Hexadecyltrimethylammonium bromide) is added to the resulting new volume sample and shaken by finger. The sample is stored in a water bath set at 65 °C for 10 minutes. Then, the total volume of phenol-chloroform-isoamyl alcohol (25: 24: 1) is added to the sample and rinsed with a rotator for 15 minutes. After shaking, centrifugation is performed at 16000 rpm for 3 minutes at 3 °C. After centrifugation, the supernatant is transferred to a sterile eppendorf and after 10 % CTAB of volume is added, it is allowed to stand for 10 minutes in a water bath set at 65 °C. An equal volume of chloroform-isoamyl alcohol (24:1) is added to the sample removed from the water bath. After the addition, the sample is shaken with the rotator for 15 minutes and then centrifuged at 16000 rpm for 10 minutes. After centrifugation, the supernatant is transferred to the sterile eppendorf. 60 % of the sample transferred to the sterile eppendorf is transferred to isopropanol stored at -20 °C and incubated overnight at -20 °C in the freezer. The sample taken from the freezer is centrifuged at 15000 rpm at 3 °C for 10 minutes. 70 % ethanol is added to the sample that has finished centrifugation and then centrifuged at 15000 rpm for 3 minutes at 3 °C. After centrifugation, the supernatant is decanted and allowed to dry at room temperature for one hour. After removal of alcohol, 70 μl of TE (Tris-EDTA) buffer is added and 3 μl of RNAase is added and allowed to dry for one hour at room temperature. Store at -20 °C. The results of DNA isolation were shown in Figure 1 (Bozoğlu, 2014).

**Bisulfite Conversion Reaction Step**

The kit was supplied from the Promega company for the bisulfite conversion reaction and the application was carried out according to the kit protocol provided by the company.

**Sodium Bisulfite Treatment**

Many bisulfite conversion kits have been advanced for bisulfite treatment because it is hard to succeed and optimal balance between complete conversion and minimal degradation of DNA. In this present study, DNA treatment with sodium bisulfite was performed using the Methyleedge™ bisulfite conversion kit (Promega, San Luis Obispo-USA) according to the manufacturer’s protocol. Also specific primer sets were designed after the bisulfite treatment for sprE gene region. Detailed information of primer set for related genes were shown in Table 1.

| T2 Primer |  |
|-----------|--|
| Forward   | TGATATCGCGATGCCACGAA |
| Reverse   | CAATAACGCGCCAACCTCTG  |
| Product length | 565 base pair |

| T7 Primer |  |
|-----------|--|
| Forward   | GTAACAGAGGGCACCAGACC |
| Reverse   | GGCAATGGGAATCACCCTGC |
| Product length | 294 base pair |

**PCR Protocol**

Samples were prepared for as a volume of 30 PCR reactions for each reaction. Each PCR reaction was contained 16.1 μl distilled water, 3 μl 10xPCR buffer, 1.2 μl DMSO, 1.8 μl MgCl₂ solution (25mM), 3μl Forward primer, 3μl Reverse primer, 0.6 μl dNTPs (deoxynucleotide triphosphates: dATP, dGTP, dCTP, dTTP), 0.3 μl Taq DNA polymerase enzyme and 1 μl bisulfite-treated DNA.

PCR conditions were as follows: 95 °C for 2 minutes, 36 cycles of denaturing at 94 °C for 1 minute, 1 minute annealing at 58 °C (This step should prepare for each primer Tin temperature), 2 minutes and 5 minutes extension steps at 72 °C (Warzak et al., 2015).
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Digestion of PCR Products with HinfI Restriction Enzyme

Digestion of PCR products was carried out by restriction enzyme HinfI enzyme following the following steps:

- 10 μl of PCR product, 18 μl of distilled water, 2 μl of 10X buffer R (compatible buffer) HinfI and 2 μl of HinfI enzyme are added. Mix vigorously and allow to incubate for 1-16 hours.
- The restriction enzyme is kept at 65 °C for 20 minutes in order to stop the reaction.
- The restriction products are stored at + 4 °C.

After this application the digested PCR products were evaluated with the QIAxcel Advanced analysis system.

Restriction Enzymatic Digestion of PCR Products

RestrictionMapper program (http://www.restrictionmapper.org) is used to determine the restriction sites in the products to be obtained after the amplification of sprE gene region with the designed specific primer sets. Restriction sites for PCR products of sprE gene information were given in Table 2. Enzymatic digestion of PCR product was performed with HinfI. These restriction enzymes were provided from Thermo-Scientific (Waltham, MA USA).

Table 2. Restriction sites of the PCR product of the sprE gene region

| Restriction Enzyme Name | Sequence | Cut Position |
|-------------------------|----------|-------------|
| HinfI                   | GANTC    | 339,974     |

After this step the digested PCR products were analyzed by QIAxcel advanced analysis system.

Selecting the Gene Region to Be Used in the Study

In this study, the basic gene region of sprE (rssB) related to nutrient starvation was selected for the applications. The known sequence information for this gene region is shown below.

![Sequence information for sprE gene](https://www.ncbi.nlm.nih.gov/nuccore/NC_000913.3?report=fasta&from=1290242&to=1291255)

The results of PCR performed to confirm the presence of the sprE gene region in Escherichia coli WP2uvrA bacterial strain, our experimental microorganism, are shown in Figure 1.
Figure 1. Demonstration of the presence of the sprE gene region in the *Escherichia coli* WP2uvrA bacterial strain.

* A: T2 primer, B: T7 primer.

**Determination of Methylation Status Change That may Occur in Case of Carbon Starvation in Selected CpG Islets in the sprE (rssB) Gene Region by COBRA Method**

**Bisulfite-treated sprE (rssB) Gene Region TAHA2 Primer**

Sequence information of the sprE (rssB) gene region after bisulfite application and CpG islands were given Figure 2.

**Table 3. TAHA2 Primers**

| Primers | Forward | Reverse |
|---------|---------|---------|
|         | GAGGYGATTAGATTITTAGTTTTGATATTTTG | TACRACAATATCAAAACCAAACCAAACCC |

![Image](image.png)

**Figure 2.** Sequence information of the *sprE* (rssB) gene region after bisulfite application and CpG islands.

**Bisulfite Treatment and PCR Reaction**

Specific products were obtained by PCR application with Taha-2 primer specially designed for bisulfite treatment after DNA isolation studies of *E. coli* samples developed on carbon restricted medium and Nutrient Broth (NB) media. The agarose gel (%1) image of these findings is shown in Figure 3.
RESULTS AND DISCUSSION

**sprE (rssB) PCR Products Restriction Application**

Restriction application studies were performed with HinfI enzyme for sprE (rssB) PCR products. QIAxcel Advanced analysis results of this study are shown in Figure 5, Figure 5.1, Figure 5.2, Figure 5.3, Figure 5.4 and Figure 5.5.

![Figure 5](image)

Figure 5. *sprE* gene region of organism developed on NB medium COBRA gel image analysis result

* A1: Marker, A2: PCR product after bisulfite application, A3: The product after restriction application.

![Figure 5.1](image)

Figure 5.1. PCR product electrophoresis after bisulfite application for the *sprE* gene region of the organism developed on NB medium.
**Figure 5.2.** Electrophoresis of the product after restriction application for the sprE gene region of the organism developed on NB medium.

**Figure 5.3.** COBRA gel image analysis of the sprE gene region of the organism developed on carbon-restricted medium.

*A1: Marker, A2: PCR product after bisulfite application, A3: Product after restriction application*

**Figure 5.4.** PCR product electropherogram after bisulfite application for the sprE gene region of the organism developed on carbon-restricted medium.
When the research findings are examined, this region is not methylated in both of the bacterial groups grown in the carbon-rich environment and in the poor environment, so that the DNA fragment is not cut due to the C → T transformation occurring in the recognition region of the enzyme as a result of the cutting application performed with the Hinfl restriction enzyme and finally, it was concluded that the methyl profile of the CpG islet had no effect on the regulation of the gene in the case of carbon starvation due to the fact that the sprE (rssB) gene region of the E.coli WP2uvrA bacterial strain did not change due to development in the carbon-rich and poor environments.

**CONCLUSION**

In this study, it was aimed to quantitatively investigate methylation motifs that may occur in regulation of sprE (rssB) gene region which is activated as a result of nutritional starvation in model prokaryotic microorganism *Escherichia coli* by COBRA method.

For this purpose, the restriction enzyme was examined in silico applications in Hinfl restriction enzyme experiments depending on the formation of the COBRA analysis of the carbon-rich 16.CpG islets of the sprE (rssB) gene region of the *E.coli* WP2uvrA bacterial strain and the development of the methylation status.

In addition, the use of capillary gel electrophoresis technique for such sensitive applications appears to be the most efficient method of presenting the separation of both small and large molecules. Accordingly, a wide range of analytical diversity, high mass sensitivity, high efficiency separation, the ability to work with very low volumes, short analysis times, minimal use of consumables and basic device setup, such as the convenience of a number of features that highlight the use of capillary gel electrophoresis technique highlights (Karakaş ve Certel, 2006). When our findings are taken into consideration, it is clearly shown that the experimental results obtained after bisulfite applications which cause high product loss can be analyzed more precisely by using capillary electrophoresis systems.

As a result, the data obtained from our present study indicate that COBRA technique can be used effectively in prokaryotic systems in the investigation of DNA methylation profile changes due to epigenetic events, similar to the applications of many eukaryotic systems in the literature (Xiong and Laird, 1997; Goedecke et al., 2009). In this respect, our present study differs from other applications performed with eukaryotic systems and shows originality. However, it has been shown that the sensitivity of the technique can be increased by adapting the capillary electrophoresis system to the common COBRA method which is known with our present study.
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