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Investigation of the Role of *cyaA/crp* Genes of *Escherichia coli* in Metal Stress

Gülçin ÇETİN KILIÇASLAN[1,*], Özge KAYGUSUZ[2], Önder İDİL[3], Cihan DARCAN[1]

[1]*Bilecik Şeyh Edebali University, Department of Molecular Biology and Genetic Faculty of Arts and Sciences, Bilecik, Turkey*
gulcin.cetin@bilecik.edu.tr, ORCID: 0000-0002-9625-224X
cihan.darcan@bilecik.edu.tr, ORCID: 0000-0003-0205-3774
[2]*Bilecik Şeyh Edebali University, Biotechnology Application and Research Center, Bilecik, Turkey*
ozge.kaygusuz@bilecik.edu.tr, ORCID: 0000-0002-3652-4266
[3]*Amasya University, Department of Basic Education, Faculty of Education, Amasya, Turkey*
onidil@gmail.com, ORCID: 0000-0003-1744-4006

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Abstract

Adenosine 3′, 5′-monophosphate (cAMP) is an important signaling molecule. CRP, the receptor protein of cAMP, acts as the 'main' regulator for transcription factors. The CRP-cAMP complex directly controls at least 500 promoters in *Escherichia coli*. In this study, the roles of *cyaA* and *crp* genes in *E. coli* BW25113 strain under metal stress were investigated. The minimal inhibition concentration (MIC) and minimal cidal concentration (MCC) of 5 different metals (Zn, Ni, Co, Cd and Cu) on *Escherichia coli* BW25113 wild type, *cyaA* and *crp* mutant cells were determined. In addition, the effect of these metals on the survival of *E. coli cyaA/crp* mutants was determined by growth and drop plate method. According to *E. coli* BW25113 wild type, *cyaA* mutant strain was observed sensitivity in all metals except copper, whereas resistance was observed in *crp* mutant strain only to zinc metal. The roles of the *cyaA* and *crp* genes in metal stress were confirmed by completing the genes on the plasmid. As a result, the roles of *cyaA* and *crp* genes in metal resistance were revealed in this study.

Keywords: *cyaA*; *crp;* Metal stress; *Escherichia coli.*

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Metal Stresinde *Escherichia coli'nin cyaA/crp* Genlerinin Rolünün Araştırılması

Öz

Adenosin 3 ′, 5′-monofosfat (cAMP), önemli bir sinyal molekülüdür. cAMP'nin reseptör proteini olan CRP, transkripsiyon faktörleri için 'ana' düzenleyici olarak görev almaktadır. *Escherichia coli*'de CRP-cAMP kompleksi, en az 500 promotor'u doğrudan kontrol etmektedir. Bu çalışmada *E. coli* BW25113 suşunda *cyaA* ve *crp* genlerinin metal stresi altındaki rolleri araştırılmıştır. *Escherichia coli* BW25113 yabanıl tip, *cyaA* ve *crp* mutant hücrelerinin 5 farklı metale (Zn, Ni, Co, Cd ve Cu) karşı minimal inhibisyon konsantrasyonu (MİK) ve minimal sidal konsantrasyonu (MSK) belirlenmiştir. Ayrıca bu metal stresinin *E. coli cyaA/crp* mutantlarının yaşamı üzerine etkisi büyüme ve petri damlatma yöntemi ile belirlenmiştir. Çalışmada *E. coli* BW25113 yabanıl tipe göre, bakır hariç tüm metallerde *cyaA* mutant suşunda duyarlılık gözlenirken, sadece çinko metalinde *crp* mutant suşunda dirençlilik gözlenmiştir. *cyaA* ve *crp* genlerinin metal stresindeki rolleri, genlerin plazmit üzerinde tamamlanması yapılarak doğrulanmıştır. Sonuç olarak; bu çalışmada *cyaA* ve *crp* genlerinin metal direncindeki rolleri ortaya konulmuştur.

Anahtar Kelimeler: *cyaA*; *crp*; Metal stresi; *Escherichia coli.*

1. Introduction

Metals are very important as they serve as structural or catalytic components of living organisms [1, 2]. Many important biological processes in living organisms, including respiration, photosynthesis, and nitrogen fixation are dependent on metal ion co-factors [3]. However, metals function as redox centers of metalloproteins, such as cytochromes and iron sulfur proteins, which play a vital role in electron transport [4]. Since transition metals are often in the oxidized state, they also play a role as electron carriers [5].

The metal concentration in the environment is gradually increasing due to various reasons such as the progress of the industry in the world, the incorrect discharge of waste products, and direct throw to water and land areas. With this increase, heavy metals such as copper, cadmium, lead, zinc, nickel, mercury, and chromium accumulated in nature have become one of the most important environmental problems affecting life on land and water [6-8]. Despite their essential roles in cellular reactions, metals exhibit toxicity when their concentrations exceed a certain level [9]. Excess metal ions catalyze unwanted reactions and biomolecular damage. It also leads to improper metal binding to random regions of metalloproteins or other proteins, resulting in

inactivation or inappropriate allosteric effects [10-12]. Metals cannot be synthesized and metabolized inside the cell. Since metals can be beneficial as well as toxic, the proper arrangement of metals in the cell is extremely important for bacteria [3, 13].

Bacteria have complex metal homeostasis mechanisms to maintain the delicate balance between the amount of metal required and toxicity [14]. These mechanisms are regulated by metal-sensitive transcription factors (metal sensor proteins and metalloregulators) that detect the level of bioavailability of a particular metal type in the cell and then regulate the transcription of genes associated with that metal [3, 15-17]. When the extracellular concentration of a particular metal increases in bacteria, the first "checkpoint" is to limit metal entry to ensure metal homeostasis and prevent cellular damage. It regulates this by reducing the expression of genes that allow metal entry [3]. For example, one of the regulators induced by copper in *E. coli* is the outer membrane protein ComC (YcfR). In the presence of high copper, the increase of *comC* expression in the cell prevents copper from being taken into the periplasmic space from the external environment [18]. Chelating or precipitation of soluble metal ions in the extracellular environment is another way to limit metal entry [19]. Also, another way to protect bacteria from metals is to reduce the toxicity of metals by using detoxification means. For example, Cue regulon in *E. coli* is responsible for copper detoxification. CueR, the main regulator of this system, is responsible for cytoplasmic Cu detection. CueO, a multiple copper oxidase enzyme regulated by CueR, oxidizes Cu^{+} to divalent Cu^{2+}, which cannot pass through the inner membrane. Thus, it helps protect periplasmic proteins [20, 21]. In addition, bacteria resist metals by increasing the excretion with many pulse-type pumps such as P1b type ATPase, resistance nodulation-division (RND) carrier, cation diffusion facilitators (Cation Diffusion Facilitato (CDF)), ABC carriers [22]. For example, in *E. coli*, Zn^{+2} and Cu^{+} are transported from the cytoplasm to the periplasm by P1b-type ATPases, which are ZntA and CopA proteins, respectively [23]. While RND complexes share a common structure in Gram negative bacteria, they are diverse for different metals. For example, in *Pseudomonas putida*, CzcCBA system provides resistance against Zn^{2+}, Cd^{2+} and Pb^{2+} [24], while in *Caulobacter crescentus*, CzrCBA and NczCBA systems are involved in Cd, Zn, Ni and Co transport, respectively [25]. CDF are mostly flow pumps that remove bivalent metal ions such as Zn^{2+}, Co^{2+}, Cd^{2+}, Ni^{2+} and Fe^{2+} from cells [26, 27]. In *E. coli,* the ZitB and YiiP antiporter pumps Zn^{2+} against H^{+} [28, 29]. However, nonspecific multidrug flow pumps have also been shown to mediate the removal of a wide variety of compounds and molecules, including metals [30].

In *E. coli*, the *cyaA* and *crp* genes encode adenylate cyclase and cAMP receptor protein, respectively [31]. Adenosine 3′, 5′-monophosphate (cAMP) is an important signaling molecule

found in many organisms. CRP, the receptor protein of cAMP, is known mostly to play a general regulatory role in carbon catabolism in *E. coli*. That is, in the absence of easily metabolized carbon sources such as glucose, the adenylate cyclase enzyme is activated by producing cyclic AMP (cAMP) from ATP. cAMP binds and activates CRP, which in most cases activates operons participating in the use of alternative carbon sources such as lactose and maltose [32]. In *E. coli*, CRP has a major role in global gene expression, with its effect on more than 380 promoters and 70 transcription factors [33]. For example, it contributes to the multiple roles of cAMP in processes such as the regulation of virulence phenotypes in pathogenic bacteria [34-36]. Bacterial cAMP systems also have key roles in cellular homeostasis, phototaxis, protein secretion, regulation of virulence, and biofilm formation [37]. Although cAMP and CRP play various roles in many known mechanisms, there are not many studies on their roles in metal resistance.

The CAMP signaling pathway is a highly conserved regulatory mechanism that plays an important role in a variety of essential cellular processes. Therefore, considering that *cyaA-crp* genes may have a role in metal stress, the importance of *cyaA-crp* genes in *E. coli* under metal stress was investigated in this study.

2. Materials and Methods

2.1. *E. coli* Strains used in the study

Wild type *E. coli* BW25113 and mutant strains (JW3378 and JW5702) used in this study were obtained from the Japanese National Genetics Center (Keio collection-Japan National Genetic Center), and are shown in Table 1. Stocks were prepared in Luria-Bertani (LB-Merck) broth medium containing 20% glycerol (Merck) and stored at –80°C (Panasonic) for future studies. The strains in the freezer were inoculated on the LB agar medium. Before being used in the study, antibiotic resistance properties were tested by colony PCR method.

Table 1: Wild type *E.coli* and mutant strains used in this study

Stock number of Strains	Genotype	Resource
BW25113	Wild type	Keio Collection
JW3378	BW25113 *cyaA::km*	Keio Collection
JW5702	BW25113 *crp::km*	Keio Collection
b3806	*cyaA*	Mobile plasmid pNT3 (Keio collection)
b3357	*crp*	Mobile plasmid pNT3 (Keio collection)
CD102	BW25113 *pnt3::cyaA*	This study
CD103	BW25113 *pnt3::crp*	This study

2.2. Plasmid isolation

In order to verify the role of the gene we studied, plasmids carrying the *E. coli* BW25113 *cyaA* and *crp* gene regions were first obtained. From the mobile plasmids bearing the *cyaA* and *crp* gene regions given in Table 1, a single colony was cultivated separately in 5 ml LB broth and incubated overnight at 37°C and 160 rpm in a shaking incubator. Plasmid isolation from bacterial cultures obtained after incubation was performed using Purelink Quick Mini Prep plasmid isolation kit (InvitroGen) and procedure. A part of the isolated plasmid was used for quantitation and the obtained plasmids were stored at –20°C. The amount of plasmid was determined non-quantitatively by running it in 0.8% agarose gel and comparing it with GeneRuler brand DNA Ladder mix (Thermo) marker.

2.3. Transformation

To obtain complementary cells of *E. coli* BW25113 mutant strains, *E. coli* BW25113 mutants (BW25113 *cyaA::km* and BW25113 *crp::km*) were inoculated separately in 2 ml of LB containing kanamycin at a final concentration of 25 µg/ml. The cultivated cultures were grown in a shaking incubator at 37ºC for 18 hours at 160 rpm and used as pre-culture for transformation. Mobile plasmids carrying the gene regions we studied were transferred to the relevant *E. coli* BW25113 mutant strains by transformation [38]. The next day, 100 µl of 2 M Mg^{2+}, a final concentration of 25 µg/ml, and 50 µl of kanamycin antibiotic and pre-culture are added to sterilized 9.9 ml Hanahan's broth (SOB medium) until the cells reach OD_{600} 0.3. It was incubated at 37ºC and 160 rpm with shaking. After incubation, each tube was divided into 2 centrifuge tubes and cooled in ice for 5 min and centrifuged at 12000 rpm for 5 min at 4ºC. One ml of 0.1 M cold $CaCl_2$ was added to the pellets and suspended. Then the tubes were kept on ice for 10 min and centrifuged again at 12000 rpm for 5 min at 4ºC. The supernatant was discarded and resuspended by adding 200 µl of 0.1 M cold $CaCl_2$ per tube and kept on ice for 30 min. The obtained 200 µl competent cell was added to 10-100 ng of 2 different concentrations of isolated plasmid and kept on ice for 45 min. Then the cell suspensions were heat shocked at 42ºC for 2 min and then on ice for 2 min. 0.8 ml of SOB medium (SOC medium) enriched with 20 mM magnesium (Merck) and 3% glucose (Merck) was added onto the cell suspension and left for 60 min incubation at 37°C. After the incubation, the cells were centrifuged at 5000 rpm for 1 min at 4ºC. Later, 100 µl SOC medium was added to the pellet and resuspended. It was spread on LB agar containing ampicillin antibiotic in a final concentration of 100 µg/ml and left for 18 h incubation. The complementary cells were confirmed by colony PCR.

2.4. Colony PCR

Colony PCR method was used to confirm the mutants and complementary cells to be used in the study. For this, the mutants obtained were suspended in 20 µl distilled water, and 1 µl was used as a mold for PCR. 7.5 µl of the reaction mixture in Table 2 was distributed on the mold, and the colony PCR was made according to the reaction conditions in Table 3. The primers used for the validation of each gene are shown in Table 4. The band sizes of the products obtained from the PCR reaction were verified with GeneRuler brand DNA Ladder mix (Thermo) by running in 1% agarose gel.

Table 2: Colony PCR reaction mixture

10x ThermaPol Reaction Buffer (Biolab)	1.0 µl
10 mM dNTP Mix	0.2 µl
10 µM forward primer (İnvitrogen)	0.5 µl
10 µM reverse primer (İnvitrogen)	0.5 µl
2 mM Mg^{+2} containing $MgCl_2$ (Biolab)	0.6 µl
0.25U Taq DNA Polymerase (Biolab)	0.05 µl
dH_2O	6.15 µl
Bacterial suspension	1 µl
Total	10 µl

Table 3: Colony PCR reaction conditions

94 ºC	3 min	1 cycle
94 ºC	1 min	30 cycle
58 ºC	1 min	
72 ºC	1 min 30 sec	
72 ºC	10 min	1 cycle

Table 4: Primers used in colony PCR reaction

Universal K1 reverse primer	5’ CAGTCATAGCCGAATAGCCT3’
BW25113::*cya* forward primer	5’ TGTTACCGTTGATTGTGGCG 3’
BW25113::*crp* forward primer	5’ GAGAAAGCTTATAACAGAGG 3’
Universal pNT3 SP6 forward primer	5’ ATTTAGGTGACACTATAG 3’
Universal pNT3 21M13 reverse primer	5’ CAGGAAACAGCTATGACC 3’

2.5. Determination of minimum inhibition concentrations (MIC) and minimum cidal concentrations (MCC) of metals

The 0.2 M stock solutions of $CuSO_4$, $NiCl_2$, $CdSO_4$, $CoCI_2$ and $ZnSO_4$ metals used in the study were prepared by dissolving in water and sterilized by filtration. MIC and MCCs of metals

were determined in round bottom 96-well microplates according to the CLSI method [39]. Each of the wild type and mutant strains was incubated in 5 ml nutrient broth medium at 37°C for 18 h with shaking. After the incubation, the absorbance of bacteria was adjusted to 0.1 at 600 nm wavelength. Then 400 µl from stock bacteria culture was prepared in 60 ml. From this culture, the 180 µl culture was added to the 96-well microplate in the first well and 100 µl in the other wells. 20 µl of the metals was added to 180 µl wells, and serial dilution was made in 12 wells. Plates were incubated at 37°C for 18 h and MIC values were determined. In addition, in order to determine the cidal effects of metals, 10 µl of culture was taken from each well where no growth was observed, and it was dropped into a medium containing nutrient agar. Then the plates were incubated at 37°C for 18 h, and their MCC values were determined. The experiments were repeated at least 3 times.

2.6. The effect of metal stress on the survival of *Escherichia coli cya*/*crp* mutants

2.6.1. Growth experiments

Each of the pure cultures obtained from *E. coli* BW25113 wild type, *cyaA::km* and *crp ::km* mutants were inoculated in 5 ml nutrient broth medium and incubated at 37°C in a shaking incubator for 18 h. After the incubation, the absorbance of the bacterial cultures was adjusted as 1.0. In order to the starting numbers to be equal (approximately OD_{600} 0.005), 75 µl of the pre-cultures adjusted to 1.0 was added to the media containing 15 ml of nutrient broth, and growth experiments were started. In metal growth experiments, ¾ of the wild-type MIC value from metal stocks was added to the growth medium. The prepared samples were left to incubate for 8 h in an agitated oven at 37°C and 160 rpm. Growth graphs were obtained by taking samples from the bacteria every 2 h and measuring their absorbance in OD_{600} in a spectrophotometer. The experiments were repeated at least independently 3 times.

2.6.2. Drop plate experiments

Plate dropping experiments were performed to see the effect of solid media on growth of metal-free and metal-based growth experiments. Also, the the number of bacteria on effect of metals was observed here. Firstly, the amount of metals used was determined in 3 different concentrations by taking into account the MIC value of wild type *E. coli* BW25113. Petri dishes containing metal at concentrations of 0.06, 0.07 and 0.08 mM for cadmium, 0.8, 0.9 and 1.0 mM for copper, 0.4, 0.45 and 0.5 mM for cobalt, 0.7, 0.8 and 0.9 mM for nickel and 0.27, 0.28 and 0.29 mM for zinc were prepared.

For plate dropping experiments, a single bacterial colony was taken from *E. coli* BW25113, *cyaA::km* and *crp::km* mutants and incubated in 15 ml NB at 37°C in a 160 rpm shaking incubator for 18 h. After incubation, the bacteria cultures were adjusted to an absorbance of OD_{600} 1.0. 1 ml of the adjusted samples was centrifuged and washed twice with Ringer's (Merck) solution. 100 µl of the obtained bacterial suspension was taken, and the final cell count was adjusted between 10^1 and 10^7 cfu/ml by diluting a series of 1/10 with Ringer's solution. Later, those with cell numbers between 10^3 and 10^7 cfu/ml from these tubes were dropped to metal-free (control) and metal-containing Nutrient Agar media as 10 µl and incubated at 37°C for 18 h. After the incubation, bacterial colonies formed in the plate were photographed with SYNGENE G: Box Chemi XRQ device. The experiments were repeated at least 3 times.

2.7. Complementation tests

At the end of the studies, complementation tests were carried out to verify the genes found to have roles according to the significance test performed with IBM SPSS 21. In these tests, the relevant gene region, which is mutant in the main chromosome, was added to the mutant strains by adding on pNT3 plasmid and *E. coli* BW25113 pnt3::*cyaA* and *E. coli* BW25113 pnt3::*crp* cells were obtained. In order to induce the pNT3 plasmid in the completed cells, IPTG (isopropyl β-D-1-thiogalactopyranoside) (Sigma) was added to the media used in the experiments at a final concentration of 1 mM. The experiments were repeated at least 3 times.

3. Results

3.1. Obtaining complementary cells

Confirmation of cells obtained as a result of transforming *cyaA* and *crp* genes into cells with mutants in the main chromosome by means of pNT3 plasmid was performed by colony PCR method. The agarose gel image of the colony PCR products made using the traditional SP6-21M13 primer of the pNT3 vector is shown in Fig. 1. According to the PCR results, samples 1, 2, and 3 are *E. coli* pnt3::*cyaA* cells with a size of 2611 bp, while samples 4, 5, and 6 are positive for *E. coli* pnt3 :: *crp* cells with a size of 697 bp. Among these cells, number 2 for *E. coli* pnt3::*cyaA* and number 6 for *E. coli* pnt3::*crp* were used in validation studies.

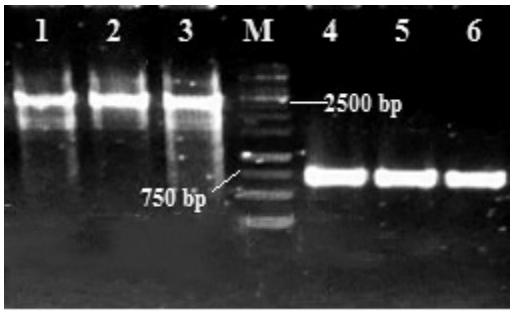

Figure 1: The colony PCR results of *E. coli* pnt3::*cyaA* and pnt3::*crp* complement strains obtained as a result of transformation. M: 1 kb DNA Ladder, 1-3. *E. coli* pnt3::*cyaA* 4-6. *E.coli* pnt3::*crp*

3.2. Minimum inhibition concentration (MIC) and minimum cidal concentration (MCC) values

Minimal inhibition concentration (MIC) and minimum cidal concentration (MCC) values of metals belonging to *E. coli* wild type and mutant strains are shown in Table 5. According to the determined results, the MIC values of *E. coli* wild type are 468 µg/ml for copper (Cu) and 30 µg/ml for cobalt (Co), and they are the same as for mutant strains. While MIC values of Ni metal did not differ between wild type and mutant strains, when MIC values of Cd metal were compared, MIC values of mutant bacteria (145 µg/ml) were two times higher than wild type MIC values (72 µg/ml). When the MIC values of Zn metal were compared, it was determined that it was 180 µg/ml in wild type *E. coli*, 135 µg/ml in *cyaA::km* and 360 µg/ml in *crp::km.* According to these results, it is seen that the *crp* mutant is sensitive while the *cyaA* mutant is resistant compared to the wild type *E. coli*. It was observed that the determined MCC results were higher than the MIC values, and the metals had growth inhibitory properties at MIC values.

Table 5: Minimum inhibition concentration (MIC) and minimum cidal concentration (MCC) values of wild-type BW25113 and *cyaA/crp* mutant strains used in the study

Metal	**MIC (µg/ml)**			**MCC (µg/ml)**		
	Wild-tip BW25113	BW25113 *cya::km*^{r}	BW25113 *crp:: km*^{r}	Wild-tip BW25113	BW25113 *cya:: km*^{r}	BW25113 *crp:: km*^{r}
$CuSO_4$	468	468	468	625	625	625
$NiCI_2$	493	329	493	986	493	986
$CdSO_4$	72	145	145	96	193	193
$CoCI_2$	30	30	30	30	60	60
$ZnSO_4$	180	135	360	720	540	1078

3.3. Growth experiments

E. coli wild type, mutant and complement bacteria were observed to grow for 8 h at 160 rpm in nutrient broth medium that does not contain metal and contains metal at the rate of the

wild type MIC value and growth graphs are given in Figs. 2-7. It was shown in Fig. 2 that the growth of *E. coli* wild type and mutant cells in medium without metal is very similar with each other and approximately OD_{600} is between 1.4-1.6.

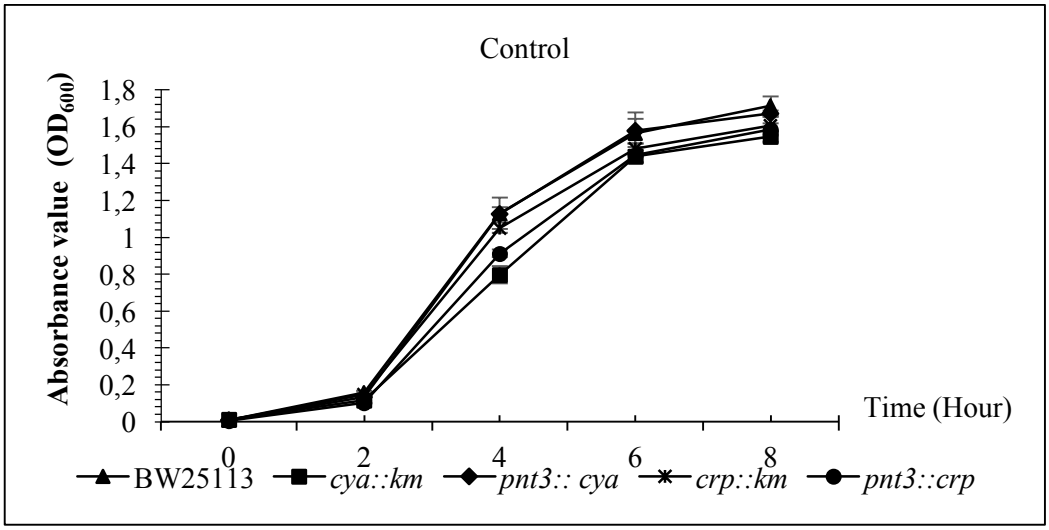

Figure 2: Growth of *E. coli* BW25113, *cyaA::km*^{r}, pnt3: *cyaA*, *crp::km*^{r} and pnt3 :: *crp* strains in metal-free medium. (*) ($p < 0.05$)

When the growth graphs in copper containing medium are examined in Fig. 3, it is seen that there was no a difference between wild type and mutant cells, and it grows up to OD_{600} 1.0. It was seen at the growth graphs obtained in the medium containing nickel metal that the wild type and the *crp::km* mutant grew close to each other, grew up to 0.5, while the *cyaA::km* mutant was more affected by nickel metal, and can grow up to 0.1. Therefore, it has been determined that the *cyaA* gene has an important role in growth in the presence of Ni (Fig. 4).

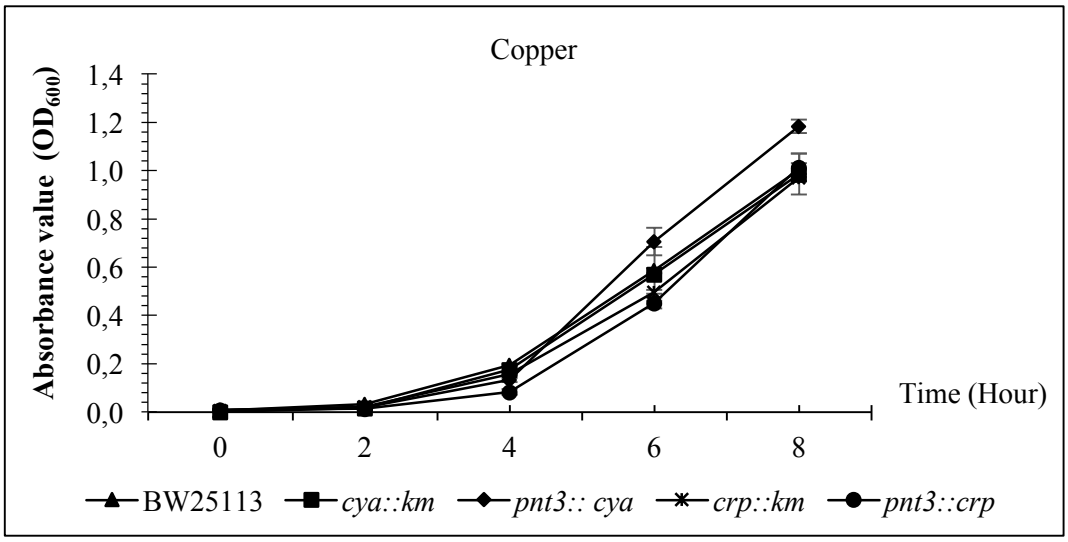

Figure 3: Growth of *E. coli* BW25113, *cyaA::km*^{r}, pnt3:: *cyaA*, *crp::km*^{r} and pnt3 :: *crp* strains in the presence of copper (Cu) metal. (*) ($p < 0.05$)

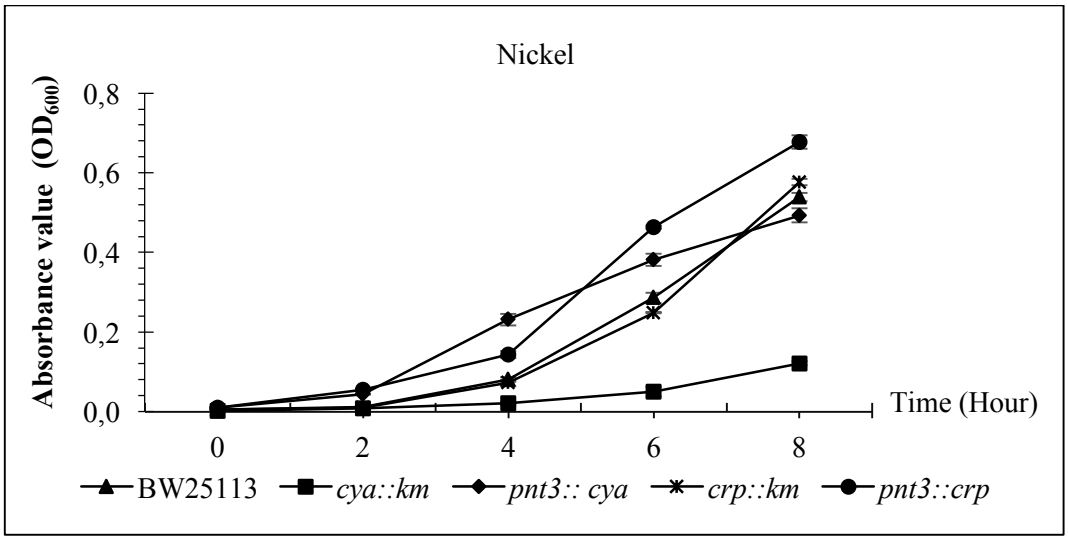

Figure 4: Growth of *E. coli* BW25113, *cyaA*::km^r, pnt3::*cyaA,* crp::km^r and pnt3::*crp* strains in the presence of Nickel (Ni) metal. (*) ($p < 0.05$)

When we look at the growth profiles in the medium containing cadmium metal, as seen in Fig. 5, the *crp::km* mutant grew up to 1.0 and partially affected as same as the wild type, which grows up to OD_{600} 1.2, while the *cyaA::km* mutant is highly affected by the cadmium metal. It has grown up to 0.7. The positive role of the *cya* gene in the presence of Cd was confirmed by complement tests.

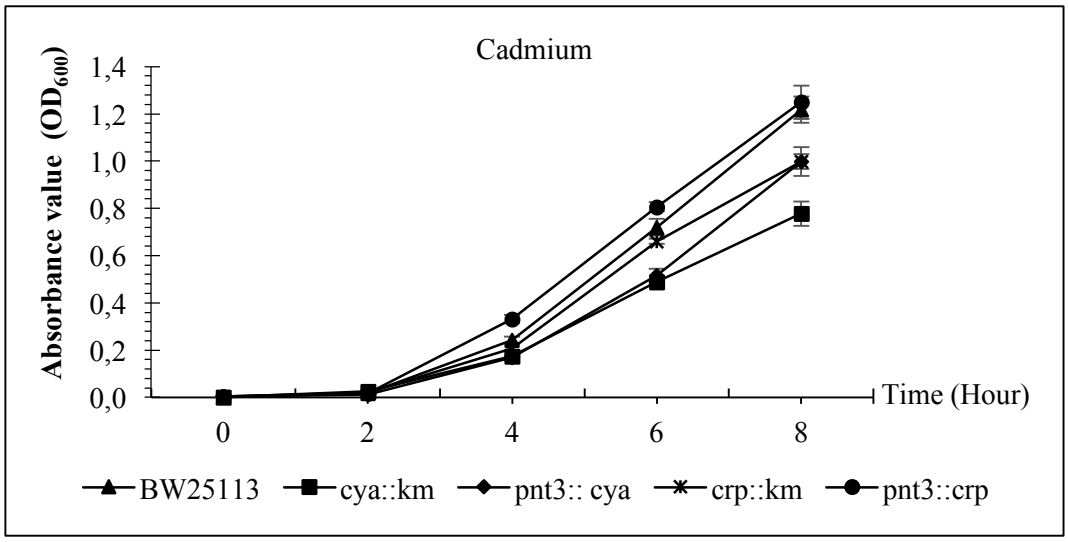

Figure 5: Growth of *E. coli* BW25113, *cyaA::km*^{r}, pnt3: *cyaA, crp::km*^{r} and pnt3*::crp* strains in the presence of cadmium (Cd) metal. (*) ($p < 0.05$)

As seen in Fig. 6, wild type and *crp::km* mutant grew close to each other (OD_{600} 0.6), while the *cyaA::km* mutant grew up to OD_{600} 0.3, and it was more sensitive to metal. Since the presence of the *cyaA* gene product induced in complement cells enables the cell to grow better, the role of this gene in the presence of Co has been confirmed.

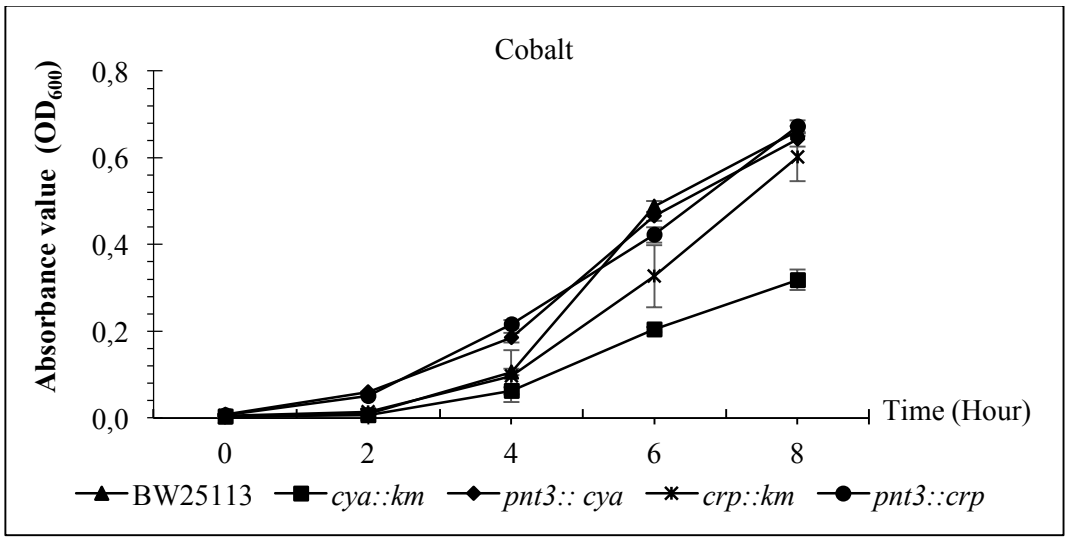

Figure 6: Growth of *E. coli* BW25113*, cyaA::km*^{r}, pnt3: *cyaA, crp::km*^{r} and pnt3*::crp* strains in the presence of cobalt metal. (*) ($p < 0.05$)

When the growth profiles of the wild-type and mutant strains in the zinc metal-containing medium in Fig. 7 were seen, it was observed that the *crp::km* mutant can grow up to 1.4 while the *cyaA::km* mutant can grow up to 0.8 compared to the wild-type growing up to OD_{600} 1.2. It has

been observed that the mutant is more affected by the metal and can grow to OD_{600} 0.8 (Fig. 7). It has been determined that cell growth is restored by the *cyaA* gene induced in complemented cells.

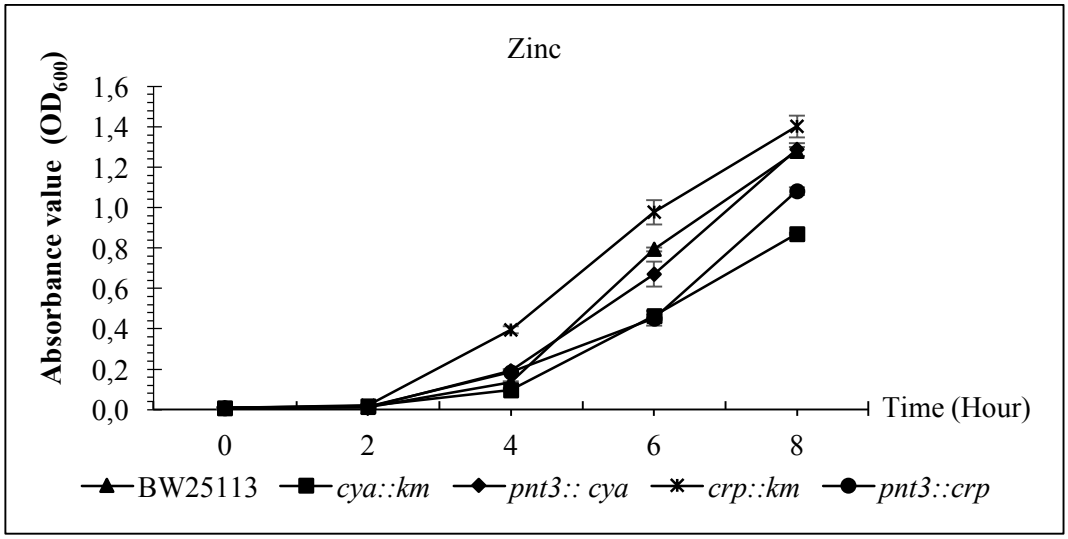

Figure 7: Growth test results of *E. coli* BW25113, *cyaA::km*^{r}, pnt3::*cyaA, crp::km*^{r} and pnt3::*crp* strains in the presence of zinc metal, (*) ($p < 0.05$)

3.4. Drop plate experiments

The effect of metal on different cell numbers was determined by plate dropping experiments of *E. coli* BW25113, *cyaA::km*^{r}, pnt3 :: *cyaA, crp ::km*^{r} and pnt3 *::crp* strains. As seen in Fig. 8A, there is no difference between the growth of wild type BW25113 and mutant cells in a metal-free plate dish. However, in plate dishes containing 1.0 mM copper, the highest concentration studied, the mutants show similar growth as the wild type (Fig. 8B). This result also supports the results in growth experiments.

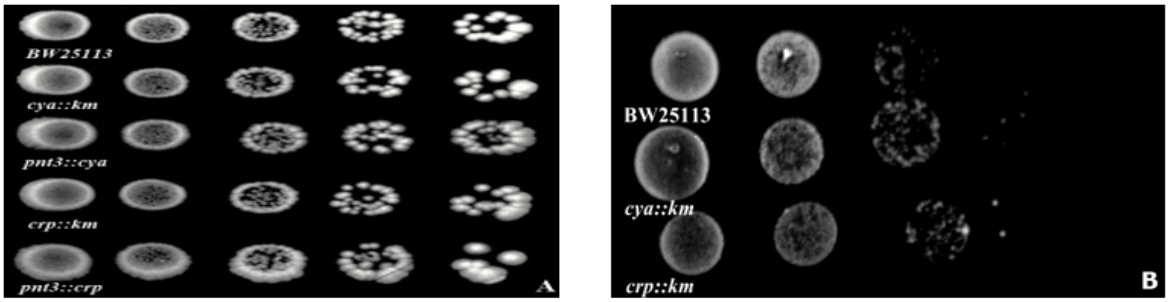

Figure 8: Plate dropping test results of *E. coli* BW25113, *cyaA::*km^r, pnt3: : *cyaA*, *crp::km*^{r} ve pnt3::*crp* strains. A: Metal free NA, B: NA with 1.0 mM Cu

It was seen in Fig. 9A that *crp::km*^{r} cells developed similar to *E. coli* BW25113 according to the dropping experiments performed on 0.7, 0.8 and 0.9 mM nickel-containing plate. However, it was seen in Figure 9B that *cyaA::km*^{r} cells were more affected and as a result of the expression of the complementary cell and the product of the *cyaA* gene on the plasmid again behaved like a wild type. As a result, when the *cyaA* gene is made mutant, the survival rate of the bacteria decreases in the presence of Ni, and it is seen that sensitivity occurs.

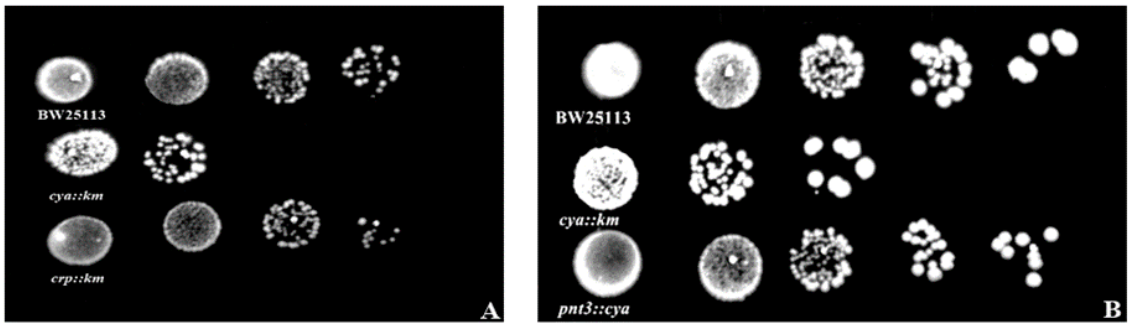

Figure 9: Plate dropping test results of *E. coli* BW25113, *cyaA::*km^r, pnt3::*cyaA*, *crp::km*^{r} ve pnt3::*crp* strains. A: NA containing 0.9 mM Ni, B: NA containing 0.8 mM Ni

According to the results of dropping on plate dishes containing 0.07 mM cadmium metal, the effect of the metal on *crp::km*^{r} cells was similar to *E. coli* BW25113, while its effect on *cyaA::km*^{r} cells was higher in Fig. 10A. Therefore, it has been determined that knockout of the *cyaA* gene causes sensitivity in the life of *E. coli* in the presence of Cd. This sensitivity indicates that *cyaA* as a role to live in the presence of Cd. The effect of the metal on the *cyaA::km*^{r} cells was again seen to resemble the wild type upon gene completion (Fig. 10B).

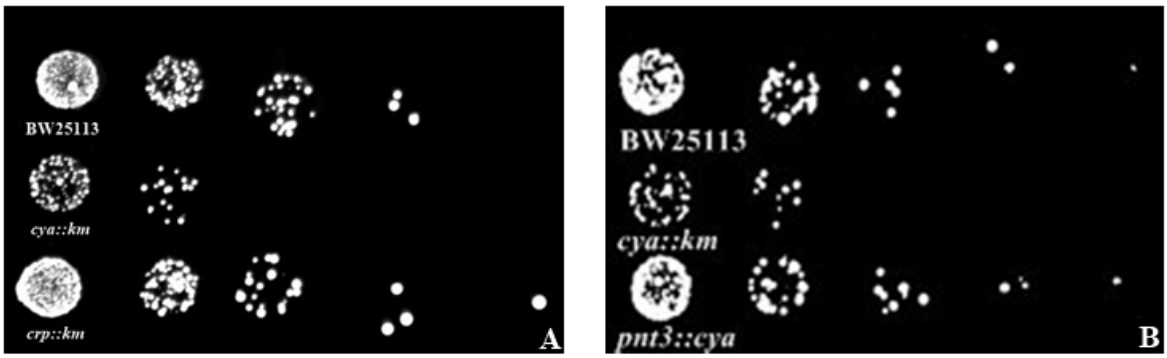

Figure 10: Plate dropping test results of *E. coli* BW25113, *cyaA::*km^r, pnt3::*cyaA*, *crp::km*^{r} ve pnt3::*crp* strains. A: NA with 0.07 mM Cd, B: NA with 0.07 mM Cd

According to the results of plate dropping with cobalt metal, *cyaA*::*km*^{r} strains were the most sensitive, while *crp*::km^{r} cells were affected similarly to the wild type (Fig. 11A). It was determined that the *cyaA* gene product is necessary for life in the presence of Co, and the cell's sensitivity increases when it is not. By using pnt3::*cyaA* complementary cell in the complement tests, it was determined that the effect of the metal against the cell was reduced and similar to the wild type (Fig. 11B).

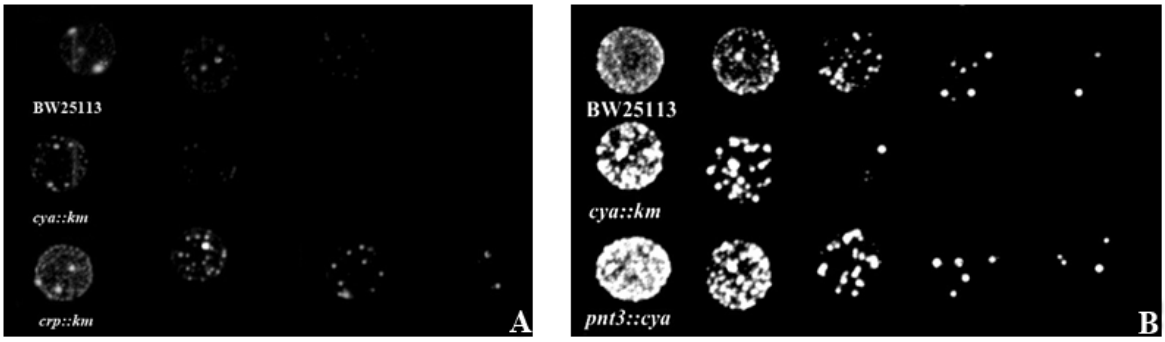

Figure 11: Plate dropping test results of *E. coli* BW25113, *cyaA*::km^{r}, pnt3: : *cyaA*, *crp*::*km*^{r} ve pnt3::*crp* strains. A: NA containing 0.50 mM Co, B: NA containing 0.40 mM Co

According to the results of plate dropping containing 0.27, 0.28 and 0.29 mM zinc, *cyaA*::*km*^{r} strains were the most sensitive to metal while *crp*::*km*^{r} cells were the most resistant (Fig. 12A-C). Performing these results with the completed cells was confirmed by affecting the cells as wild type again.

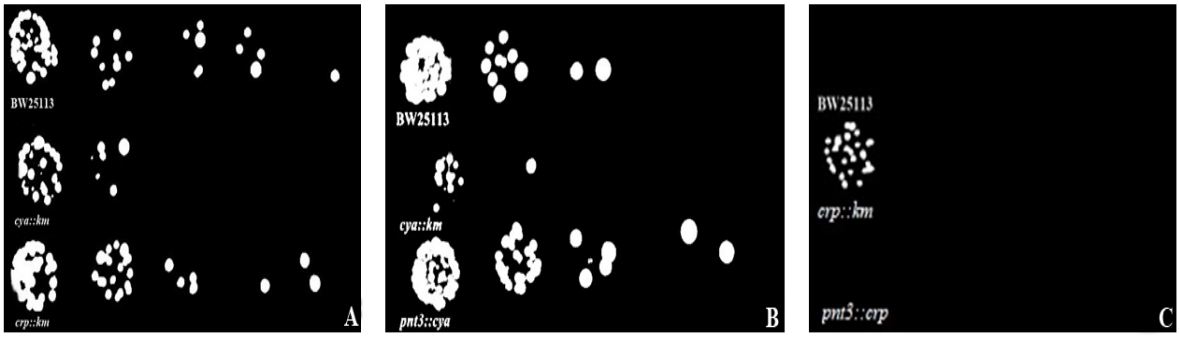

Figure 12: Plate dropping test results of *E. coli* BW25113, *cyaA*::km^{r}, pnt3: : *cyaA*, *crp*::*km*^{r} ve pnt3::*crp* strains A: NA with 0.27 mM Zn, B: NA with 0.28 mM Zn, C: NA with 0.29 mM Zn

4. Discussion

Metals are essential trace nutrients for the growth of living organisms. However, metals are toxic at high concentrations. For this reason, maintaining the metal balance in the cell is very

important for life. Bacteria possess complex metal homeostasis mechanisms to maintain the delicate balance between this metal starvation and toxicity [41]. Determining the genes involved in these complex regulatory systems and the functions of these genes is very important in terms of both bacterial life and fight against bacteria.

In this study, according to the MIC results of the copper, nickel, and cobalt metals, no significant difference was observed in the *cyaA* and *crp* mutants when compared with the wild type, while the MIC results of cadmium showed that the mutants were twice resistant according to the wild type. When MIC results of zinc metal were compared, it was determined that *cyaA* mutants were susceptible, while *crp* mutants were resistant according to wild type. The MCC results also support the MIC results of copper, cobalt, zinc, and cadmium metal. However, it was observed that the *cyaA* mutant, which did not show a significant difference in MIC results of nickel metal, was more sensitive than wild type in MCC results. This sensitivity was evident in growth experiments in the presence of nickel.

In our study, according to the results of plate dropping and growth experiments obtained with copper metal, cell life is not affected by deletion of *cyaA* and *crp* genes. According to these results, it can be thought that the *cyaA* and *crp* genes in *E. coli* do not have a role in protecting the copper metal balance in the cell. However, it is known that *E. coli* has many regulator systems in charge of ensuring the use of copper under changing environmental conditions and to protect it from its toxicity [14]. In *E. coli*, Cue and Cus, two important systems responsible for detoxification of excess copper, adjust the intracellular copper level according to cellular demand [18-42]. The CopA protein induced by CueR, which detects copper in the cell, enables the transfer of copper in the cytoplasm to the periplasm, while the CusCFBA pump induced by the CusR/S system, which detects the copper in the periplasm, ensures that the copper is expelled to the outside environment [18, 21, 43]. Our study shows that *cyaA* and *crp* genes do not play a role in maintaining copper metal balance, since they do not affect the life of mutant cells under copper stress.

In the results of cobalt, zinc, nickel, and cadmium petri dripping and growth experiments performed in our study, it was observed that *cyaA* mutant cells were more sensitive than wild type. cAMP is an intracellular messenger molecule synthesized from ATP by the adenylate cyclase enzyme CyaA, and its concentration in the cell changes due to various reasons [44]. Changes in intracellular cAMP concentration are known to be detected by the transcription factor CRP, and the resulting CRP-cAMP complex directly controls at least 500 promoters in *E. coli* [45]. Therefore, according to the results obtained in our study, it was determined that *cyaA* mutant cells exposed to nickel, cobalt, zinc, and cadmium metal stress were more affected than wild type.

In the literature, it has been reported that cAMP is degraded by enzymes stimulated by the increase in the amount of Ca^{2+}, Fe^{2+} or Co^{2+} in the cell, and thus the amount of cAMP in the environment decreases [46]. In our study, it can be stated that the deletion of the *cyaA* gene, which acts as the 'main' regulator for transcription factors, is sensitive to the inhibition of cAMP production from ATP and in the presence of Ni^{2+}, Cd^{2+} and Zn^{2+} metals in the cell, because it cannot show a regulatory role on the genes required for resistance. In this case, it can be stated that *cyaA* mutants control genes associated with Ni, Cd, and Zn resistance, and their regulation of these genes should be studied.

Another situation obtained in our study was that although there was no difference between the wild type and *crp* mutant cells in the presence of cobalt, copper, nickel, and cadmium metals, it was determined that mutant cells were more resistant under zinc stress. Sun, H., et al. (2011), sequencing of *crp* genes were performed in Fe^{3+} resistant *E. coli* K-12 MG1655 bacteria, and it was determined that two mutations occurred on the gene. In addition, in the study, it was determined that different metals caused mutations on different genes and as a result, resistant strains were obtained [47]. Because Zn^{2+} forms more stable complexes than other basic metal ions, zinc toxicity is thought to be due to improper binding of metalloproteins to metals. Therefore, intracellular concentrations must be tightly regulated [48, 49]. The presence of Zn^{2+} in bacteria is detected by various transcription factors sensitive to zinc inside the cell [50, 51]. Among these transcription factors, Zur [52-55] and AdcR [56-58] to increase zinc uptake; *zntR*, *smtB*, *ziaR*, and *czrA* genes [59-62] are included to increase the excretion and intracellular secretion of zinc. It is also known to play a role in many global regulatory mechanisms such as RpoS, which are found to be involved in metal stress [63-66]. In the study, it is thought that the *crp* gene mutant cells in the presence of zinc metal are more resistant than the wild type, either because they prevent excess zinc passage into the cell as a result of affecting the genes associated with zinc uptake, or it may be due to the excretion of excess zinc in the cell by affecting the genes that play a role in zinc excretion mechanisms.

5. Conclusion

In this study, it has been found that there is a relationship between *cyaA*-*crp* genes and resistance or sensitivity to Ni, Cd, Co, Cu, and Zn metals. It has been determined that adenylate cyclase has an important role in life against metals since it is sensitive to metals except Cu, especially in the absence of adenylate cyclase, the product of *cyaA gene*. In this case, the roles of genes such as *zntR, cueO, cueR,* and *nikR,* which are directly involved in the intracellular regulation of metals in *E. coli*, should be investigated in future studies.

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