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Recombinant Production of *E. coli* NAD+-dependent DNA ligase as a Target for Antibacterial Drug Discovery

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ABSTRACT
The increase in the frequency of drug resistance in bacterial infections has led to the research of new antibacterial agents targeting new mechanisms. Many of the functions of NAD+-dependent DNA ligase have made it a remarkable target for antibacterial drug discovery. *Escherichia coli (E. coli)* NAD+-dependent DNA ligase is presented as a potential target due to its unique substrate specificity compared to the ATP-dependent human DNA ligase. In this study, it was aimed to produce and purify the *E. coli* NAD + dependent DNA ligase enzyme, which is frequently used in antibacterial drug discovery. The *E. coli* DNA ligase gene sequence was cloned into pTOLT vector system. *E. coli* DNA ligase enzyme was purified after the production in *E. coli BL21 (DE3) pLysE* cells. It was clearly demonstrated by the activity test that the DNA ligase enzyme produced in this study can ligate the DNA fragments. As a result, it was revealed that the effect of candidate inhibitors can be studied simply on the enzyme.

Research Article

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Antibakteriyel İlaç Keşfi İçin Bir Hedef Olarak *E. Coli* NAD+-Bağımlı DNA Ligazın Rekombinant Üretimi

ÖZET
Bakteriyel enfeksiyonlarda ilaç direnci frekansındaki artış, yeni mekanizmaları hedefleyen yeni antibakteriyel ajanların araştırılmasına yol açmıştır. NAD+-bağımlı DNA ligazın fonksiyonlarının bir çoğu, onu antibakteriyel ilaç keşfi için dikkate değer bir hedef haline getirmiştir. *Escherichia coli (E. coli)* NAD+ bağımlı DNA ligazı, ATP bağımlı insan DNA ligazı ile karşılaştırıldığında, benzersiz substrat özgüllüğü nedeniyle potansiyel bir hedef olarak görülmektedir. Bu çalışmada antibakteriyel ilaç keşiflerinde sıklıkla kullanılan *E. coli* NAD+ bağımlı DNA ligaz enziminin, yüksek miktarda ve saflıkta üretilmesi amaçlamıştır. *E. coli* DNA ligaz gen dizisi, pTOLT vektör sistemine klonlanmıştır. *E. coli* DNA ligaz enzimi, *E. coli BL21 (DE3) pLysE* hücrelerinde üretildikten sonra saflaştırılmıştır. Bu çalışmada üretilen DNA ligaz enziminin DNA fragmanlarının ligasyonunu sağlayabildiği, aktivite testi ile açıkça gösterilmiştir. Sonuç olarak, bu enzim üzerinde aday inhibitörlerin etkisinin basitçe incelenebileceği ortaya koyulmuştur.

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INTRODUCTION
DNA ligase is important in DNA repair and replication (Cardona-Felix et al. 2010). DNA synthesis in one strand may be continuous in DNA replication, while synthesis is not continuous in the other strand. DNA ligase is crucial for joining Okazaki fragments and fulfilling DNA synthesis (Timson et al. 2000). Nicks caused by environmental factors such as radiation, oxidants and alkylating agents which are repaired by the activity of the DNA ligase (Crut et al. 2008). DNA ligase is an essential protector of genome integrity (Odell et al. 2000). Various organisms such as *E. coli* (Wilkinson et al. 2005), *Mycobacterium tuberculosis (M. tuberculosis)* (Srivastava et al. 2007),

Staphylococcus aureus (S. aureus) (Kaczmarek et al. 2001) have NAD^+-dependent DNA ligase. T7 bacteriophage, *Haemophilus influenzae (H. influenzae*) (Doherty et al. 1996), *Staphylothermus marinus (S. marinus)* (Seo et al. 2007) have ATP-dependent DNA ligase. In addition, all known eukaryotic DNA ligases are ATP dependent (Wilkinson et al. 2001). Bacterial genomes sequenced so far contain at least one NAD^+-dependent DNA ligase. NAD^+-dependent ligase has been cloned from various bacterial sources and sequenced. These sequences appear to be significantly similar and all have almost the same size (75 kDa) (Timson et al. 2000). These enzymes have two functional domains: the C terminal domain is the domain of binding to DNA. It is seen that a zinc ion is bound in the C terminal domain of these enzymes. The N terminal domain is responsible for the self-adenylating reaction and NAD^+ binding (Wilkinson et al. 2001). The DNA sequence of the DNA ligase gene of a wide range of bacterial species (Jonsson et al. 1994; Lauer et al. 1991; Subramanya et al. 1996; Timson et al. 2000) has been identified, and some conserved regions have been determined and compared with the discovery regions in *E. coli*. Alignment of amino acid sequences displays that *S. aureus* NAD^+-dependent *DNA ligase* has 47% and 60% amino acid identity with *E. coli* and *Bacillus stearothermophilus* NAD^+-dependent DNA ligase, respectively. It has been displayed in further studies that these DNA ligases have motifs preserved in other bacteria (Kaczmarek et al. 2001).

E. coli DNA is a protein encoded by the LigA gene, the enzyme has 671 amino acids and its molecular weight is 73,690 kDa. Wilkinson et al. (2005) found that *E. coli* LigA protein has contains zinc-finger, oligomer-binding (OB) h-barrel, helix–hairpin–helix motif (HhH) and BRCT domain. In the same study, *E. coli* LigA gene was cloned into the pET16b vector. The vector contains a 10-His-Tag containing 21 extra amino acids at the N-terminal of protein. Also, it was stated that the presence of His-Tag at the N-terminal or C-terminal end did not affect enzyme activity (Wilkinson et al. 2005).

NAD^+-dependent DNA ligases are considered to be possible targets for broad spectrum antibacterial compounds (Gong et al. 2004; Kaczmarek et al. 2001; Lee et al. 2000; Sriskanda and Shuman 2002; Swift and Amaro 2009), because they are unavoidable for many basic processes of DNA metabolism, including replication, recombination processes and repairs that require re-synthesis of DNA connection of Okazaki fragments in the pathway (Timson et al. 2000). The ligase inhibitors developed are generally competitive inhibitors and can bind to hydrophobic pockets near the NAD^+ substrate binding site (Swift and Amaro 2009). They inhibit the LigA activity of *Streptococcus pneumoniae (S. pneumoniae), H. influenzae, E. coli, Mycoplasma* and *S. aureus.* Their inhibitory activities are very high (in the nanomolar range) and are selective for bacterial DNA ligase (Mills et al. 2011). There are several studies in the literature on the inhibition of *E. coli* DNA LigA. Gong et al. found that pyropyranone compounds strongly inhibited the ligation activity of *E. coli* DNA LigA (IC_{50} = 0.6 µM) (Gong et al. 2004). Dwivedi et al. have demonstrated that Quinacrine (9-aminoacidine) is a strong candidate for inhibition of *E. coli* DNA LigA (Dwivedi et al. 2008). Mills et al. have described several adenosine analogs as inhibitors of LigA. These compounds have been shown to be effective in *H. influenzae* as well as *E. coli* DNA LigA (Alomari 2019; Mills et al. 2011).

In this study, *E. coli* NAD+-dependent DNA ligase enzyme, which is frequently used in antibacterial drug discoveries, was aimed at high level production and purification. First, the *E. coli* DNA ligase gene was cloned into the pTOLT vector system and then expressed in *E. coli BL21 (DE3) pLysE* cells. The enzyme was purified by affinity chromatography and its activity was demonstrated.

MATERIALS and METHOD

Cloning of the *E. coli* DNA Ligase Gene into the pTOLT Vector System

The gene was first amplified for cloning the *E. coli* DNA Ligase gene into the pTOLT vector (Anderluh et al. 2003). PCR (polymerase chain reaction) mixture was prepared using primers of *ligABamHI*sense 5'TTTTGGATCCATGGAATCAATCGAACAAC3' and *ligAKpnI*reverse 5'TTTTGGTACCGCTACCCAGCAA ACGCAG3'.

After PCR reaction, 10 µl of PCR mixture was taken and analyzed in 1% agarose gel. The obtained PCR products were purified with the PCR products cleaning kit and digested with *BamHI* and *KpnI* restriction enzymes. The pTOLT plasmid which was used for cloning was also cut with the same restriction enzymes. *E. coli* DNA Ligase gene and pTOLT plasmid digestion by restriction enzymes were ligated with T4 DNA ligase enzyme at room temperature for 16 hours. The ligation products were transferred to *E. coli* DH5α cells and spread on ampicillin (100 µg ml^{-1}) containing LB medium. Plasmid DNA was isolated from the colonies, and diagnostic restriction digest and diagnostic PCR were performed on plasmids. The obtained products were analyzed in 1% agarose gel.

Expression of *E. coli* DNA Ligase Protein

Protein expression and purification was carried out similar to the protocol detailed in our previous work (Kuduğ et al. 2019; Kaplan et al. 2021). In summary, for protein expression, pTOLT clones were transformed into *E. coli* BL21 (DE3) pLysE and spread on LB medium containing ampicillin (100 µg ml^{-1}).

After overnight incubation, the colonies were transferred to the 4 mL LB liquid medium containing antibiotics. Then the cells were inoculated instead of 600 mL antibiotic LB liquid medium and protein expression was induced by IPTG when OD600: 0.7. The cells were collected by centrifugation 4 hours after induction and lysed by sonicator. The lysate was centrifuged, supernatant was taken and proteins were purified by affinity chromatography (Ni-NTA Agarose Resin). Each fraction taken from the column was analyzed in SDS-PAGE.

Activity Test of *E. coli* DNA Ligase enzyme

The pET28b vector was digested for 3 hours with *BamHI* and *ApaI* enzyme. After 3 hours, it was purified with the "Biobasic Gel and PCR Clean-Up" kit. The digested and purified pET28b vector was ligated with different amounts of *E. coli* DNA ligase enzyme elutions for 2 hours at 25°C.

RESULTS and DISCUSSION

Cloning of the *E. coli* DNA Ligase Gene into the pTOLT Vector System

As a result of the PCR reaction, it was observed in the 1% agarose gel that the *E. coli* DNA ligase gene (LigA) was amplified in the 2016 bp size (Figure 1).

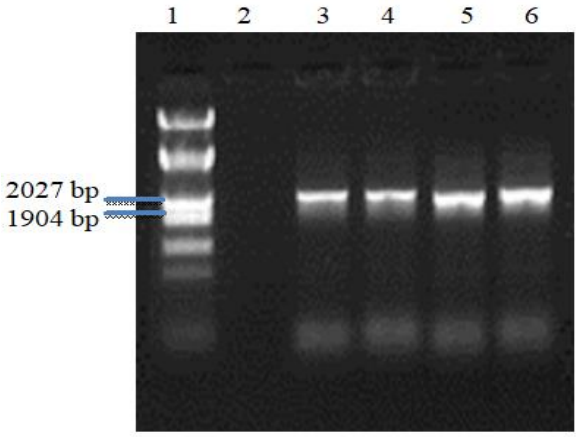

Figure 1. Analysis of the colony PCR results in 1% agarose gel. Column 1 is λDNA/EcoRI/ HindIII Marker, Columns 3-6 is 2016 bp PCR product.

Şekil 1. Koloni PCR sonuçlarının %1 agaroz jelde analizi. Sütun 1, λDNA/EcoRI/ HindIII markeridir, Sütun 3-6, 2016 bç PCR ürünüdür.

Confirmation of cloning was performed by diagnostic restriction digestion using *MluI* and *XhoI* restriction enzymes. As seen in Figure 2, it is revealed with the presence of the *E. coli* Lig A gene region around 2016 bp and the pTOLT vector around 4300 bp.

Diagnostic PCR was also performed with *ligABamHI*sense and *ligAKpnI*reverse primers using plasmid DNAs that were found to be positive in the diagnostic restriction digestion process (Figure 3).

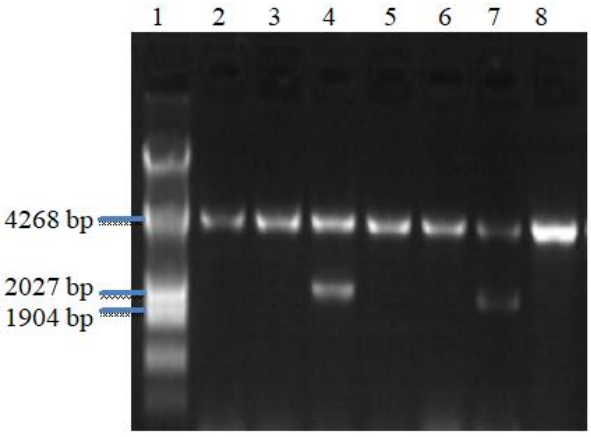

Figure 2. Analysis of the diagnostic digestion results in 1% agarose gel. Column 1 is λDNA/EcoRI/Hind III Marker, Columns 4 and 7 are plasmids containing the *E. coli* LigA gene, and columns 2, 3, 5, 6, 8 are plasmids that do not contain the *E. coli* LigA gene.

Şekil 2. Doğrulama kesim sonuçlarının %1 agaroz jelde analizi. Sütun 1, λDNA/EcoRI/ HindIII markeridir, Sütun 4 ve 7, E. coli LigA genini içeren plazmitlerdir ve sütun 2, 3, 5, 6, 8, E. coli LigA genini içermeyen plazmitlerdir.

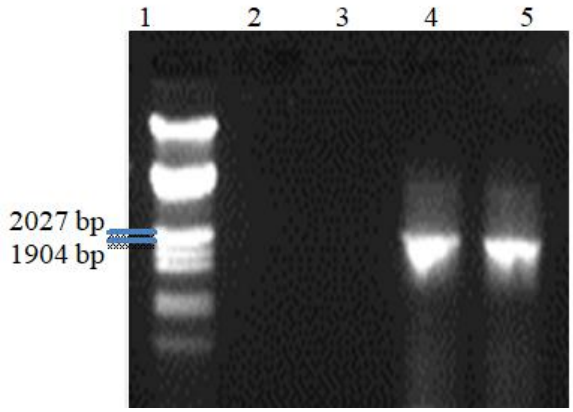

Figure 3 Analysis of diagnostic PCR results in 1% agarose gel. Column 1 is λDNA/EcoRI/HindIII Marker and columns 4, 5 are PCR products.

Şekil 3. Doğrulama PCR sonuçlarının %1 agaroz jelde analizi. Sütun 1, λDNA/EcoRI/ HindIII markeridir ve 4. 5. sütunlar PCR ürünleridir.

As a result of diagnostic PCR and diagnostic restriction analysis, *E. coli* LigA gene has been successfully cloned into pTOLT vector and, the pTOL-*E. coli* DNA Ligase plasmid was obtained (Figure 4).

Expression of *E. coli* DNA Ligase Protein

The pTOLT plasmid has a DNA sequence encoding the TolAIII peptide of 9 kDa as fusion. TolAIII is a small domain, expressed in high yields of soluble proteins in the cytoplasm of *E. coli*. The *E. coli* DNA ligase enzyme is 74 kDa in size and appears to be 83 kDa with the 9 kDa TolAIII peptide in SDS-PAGE gel (Figure 5).

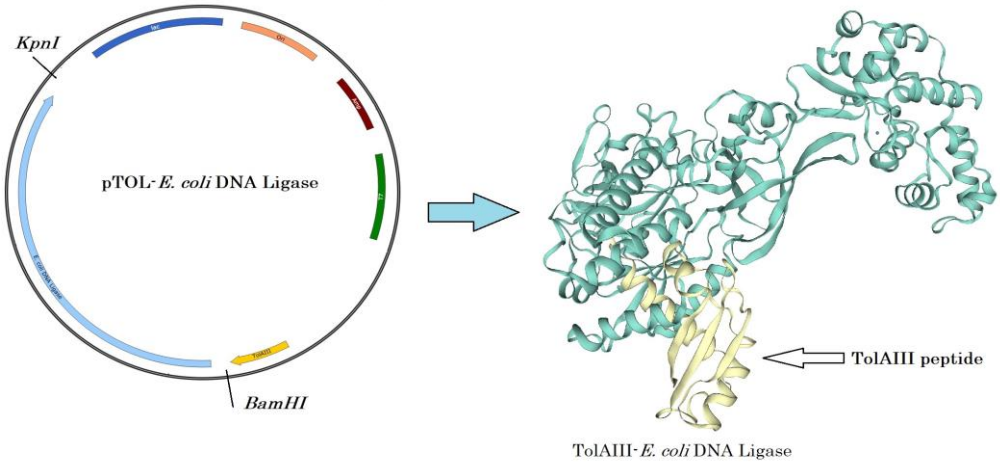

Figure 4. Plasmid map of pTOL-*E. coli* DNA Ligase construct to produce TolAIII and *E. coli* DNA Ligase fusion
Şekil 4. TolAIII ve E. coli DNA Ligaz füzyonunu oluşturan pTOL-E.coli DNA Ligaz'ın plazmid haritası

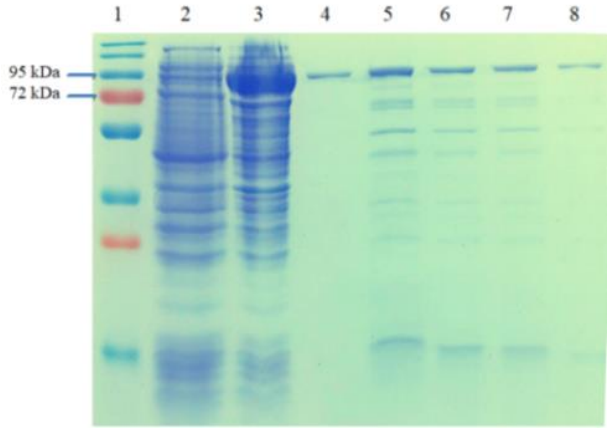

Figure 5. Analysis of the purified *E. coli* DNA Ligase protein in 12% SDS-PAGE gel. 1. Page Ruler Plus Prestained Protein Ladder 2. *E. coli BL21 (DE3) pLysE* cell lysates before IPTG addition, 3. *E. coli BL21 (DE3) pLysE* cell lysates after IPTG addition, 4-8. purified Tol-III-A-DNA Ligase fusion protein.

Şekil 5. Saflaştırılan E. coli DNA Ligaz poteininin % 12 SDS-PAGE jelinde analizi. 1. Page Ruler Plus Prestained Protein Ladder 2. IPTG ilavesinden önce E. coli BL21 (DE3) pLysE hücre lizatları, 3. IPTG ilavesinden sonra E. coli BL21 (DE3) pLysE hücre lizatları, 4-8. saflaştırılmış Tol-III-A-DNA Ligaz füzyon proteini.

The protein obtained in column 4 is> 95% purity. Protein concentration was determined as 2 mg ml^{-1} by the Bradford method.

Activity Test of *E. coli* DNA Ligase enzyme

The activity test was performed by taking different concentrations of *E. coli* DNA ligase enzyme purified by affinity chromatography. As a negative control, an Enzyme-free sample and a sample containing elution (1 µl) from *E. coli BL21 (DE3) pLysE* cells without plasmid were used. As a positive control, T4 DNA Ligase enzyme was used (Figure 6). It was clearly seen that the DNA ligase enzyme produced in this study ligated the DNA fragments. These results reveal that the effect of inhibitors on *E. coli* DNA ligase enzyme can be demonstrated simply in agarose gel electrophoresis.

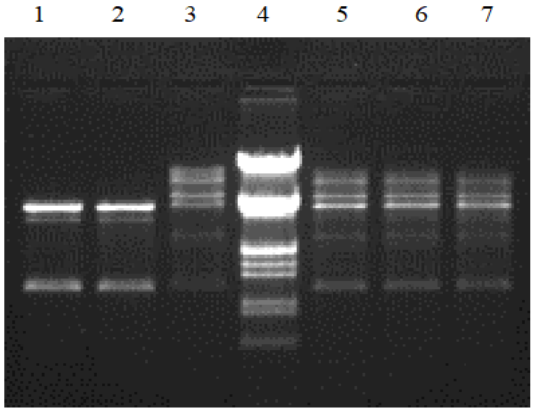

Figure 6. Analysis of the activity test of the purified protein in 1% agarose gel. 1. Enzyme-free sample. 2. Sample containing elution (1 µl) from *E. coli BL21 (DE3) pLysE* cells without plasmid. 3. Sample containing T4 DNA Ligase enzyme (1 µl). 4. λDNA/EcoRI/HindIII Marker. 5, 6, 7 are samples containing 1, 2, 3 µl of *E. coli* DNA Ligase elution, respectively.

Şekil 6. Saflaştırılan proteinin aktivite testinin % 1 agaroz jelde analizi 1. Enzim içermeyen numune. 2. Plazmid içermeyen E. coli BL21 (DE3) pLysE hücrelerinden elde edilen elüsyonu içeren örnek (1 ul). 3. T4 DNA Ligaz enzimi (1 µl) içeren örnek. 4. λDNA /EcoRI/HindIII Marker. 5, 6, 7 sırasıyla 1, 2, 3 µl E. coli DNA Ligaz elüsyonu içeren örneklerdir.

CONCLUSION

DNA ligase plays an important role in DNA recombination, replication, and repair of all living organisms. The bacterial NAD^+-dependent DNA ligase

(LigA) has high potential as a broad spectrum antibacterial target. Bacterial NAD^+-dependent DNA ligase is essential for the viability of all Gram-positive and Gram-negative bacteria tested so far, including *S. aureus, S. pneumoniae, Escherichia coli, Salmonella typhimurium, Bacillus subtilis, Salmonella enteritidis* (Dermody et al. 1979; Kaczmarek et al. 2001; Meier et al. 2008; Shuman 2009). NAD^+-dependent DNA ligases were not detected in humans, which led to the research that it could be the target for broad-spectrum antibiotics (Brotz-Oesterhelt et al. 2003;

Georlette et al. 2003; Gong et al. 2004; Kaczmarek et al. 2001; Lee et al. 2000; Sriskanda and Shuman (2002) spectrum antibiotics (Brotz-Oesterhelt et al. 2003; Georlette et al. 2003; Gong et al. 2004; Kaczmarek et al. 2001; Lee et al. 2000; Sriskanda and Shuman 2002). NAD^+-dependent DNA Ligases have low similarity with eukaryotic DNA ligase, and the need for NAD^+ as a substrate for ligase activity makes the bacterial ligase unique, thus limiting toxicity for humans (Korycka-Machala et al. 2007). Ligases are found in all three systems of life (bacteria, archaea and eukaryotes), while NAD^+-dependent enzymes are only present in bacteria (Wilkinson et al. 2001). At least one NAD^+-dependent DNA ligase is found in each bacterium. Genetic studies have demonstrated that the LigA gene is required for *E. coli* growth (Shuman 2009). NAD^+-dependent ligase (LigA) is an essential enzyme for the survival of *E. coli* and its homologous protein may be required for all bacteria (Wilkinson et al. 2001).

In this study, the *E. coli* DNA ligase gene fragment was cloned into the pTOLT vector system. The protein expression was performed in *E. coli BL21 (DE3) pLysE* cells and the protein was purified. The *E. coli* DNA ligase protein was highly expressed by fusion with the TolAIII peptide. The greatest advantage of expression in combination with TolAIII is that it provides high efficiency protein production in *E. coli* (Anderluh et al. 2003). The TolAIII peptide significantly increased the production of *E. coli* DNA ligase protein. The activity of the purified protein was confirmed by an activity test. It has been revealed that the effect of selected inhibitors can be studied simply on the enzyme we produce within the scope of this study. In addition, the produced *E. coli* DNA ligase enzyme can also be used in molecular cloning processes.

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Researchers Contribution Rate Declaration Summary

The authors declare that they have contributed equally to the article.

Conflicts of Interest Statement

Authors have declared no conflict of interest.

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