Isolation of An Erythromycin-Resistant (Putative) Gene From *Bacillus Halodurans* CM1

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**Abstract.** *Bacillus halodurans* CM1 is one of the bacterial strains from BPPT Culture Collection which had been isolated from sediments in the Cimanggu hot spring, West Java, previously. The whole genomic DNA of this strain has not yet been deeply studied despite its potential as host to produce several kinds of useful enzymes, such as xylanase, lipase, and protease. Besides the industrial enzymes, potential genes in *B. halodurans* CM1, for example antibiotic-resistant genes, could be engineered later for further purposes. Earlier experiments showed that CM1 strain has the ability to survive in the media with erythromycin, suggesting there might be genes responsible for this erythromycin resistance. This study aimed to isolate erythromycin-resistant gene from *B. halodurans* CM1 using a PCR approach. The primers used for PCR were designed based on the GenBank database of whole genome *Bacillus halodurans* C-125. The PCR product was then ligated into a pGEM-T easy cloning vector, and the recombinant vector then transformed into *Escherichia coli* DH5α. The DNA fragment with the size of 864 bp was obtained and it encoded 288 deduced amino acids. DNA sequence analysis showed that the gene had 99% similarity with gene encoding erythromycin resistance from *B. halodurans* C-125 (GenBank No access: BA000004.3, *ErmK*). There are 3 nucleotides in the *ErmK* sequence of *B. halodurans* CM1 that are different from that of another well-known strain *B. halodurans* C-125. This dissimilar pattern of sequence encodes Leu instead of Ser in 155th orders of CM1 amino acid sequence.

1. Introduction

Antibiotic resistance is a natural survival mechanism possessed by microorganisms throughout the environment [1, 2]. Bacterial resistance begins with exposure to antibiotics. Exposure to antibiotics has the opportunity to create a new strain that has the ability to be antibiotics-resistant [3]. The phenomenon of antibiotic resistance precedes the use of antibiotics [2]. Resistant bacteria will replace sensitive bacteria so that therapy with antibiotics cannot be used anymore [4, 5]. Faster resistance activity compared to the discovery of antibiotics is the basis for the surveillance of existing antibiotics [2].

The ability of bacterial resistance to antibiotics is as self-protection against the activity of other bacteria in the same environmental community. The research of antibiotic resistance genes detection...
reveals that there are a series of genes that contribute to the antibiotic resistance phenotype. A series of genes is the main factor that can encode proteins with other functions in cells or often called resistome. Gene detection can reveal the diversity of antibiotic-resistant genes possessed by bacteria. However, bacterial resistance to an antibiotic can be used in genetic engineering [6].

One of the uses of bacterial resistance to antibiotics in genetic engineering is a new cloning vector. The need for new cloning vectors is in line with the increasing number of biotechnology applications. Most gene cloning vectors in bacteria are based on multicopy plasmids which carry a marker of antibiotic resistance selection [7]. Cloning vector plasmid uses antibiotic-resistant genes found in the basic vector structure [8]. Antibiotic-resistant genes can be used as markers in direct selection of transformant colonies that carry the desired target gene [8, 9, 10]. Thus, genetic manipulation of natural isolates is needed in the manufacture of newly cloned vector plasmids that carry antibiotic-resistant genes [11].

Some groups of bacteria have a susceptibility to some antibiotics at certain concentrations. Bacillus spp. can grow on the medium with antibiotics vancomycin, gentamicin, kanamycin, erythromycin, tetracycline, streptomycin, clindamycin, and chloramphenicol [12]. In the earlier experiments, the susceptibility of B. halodurans CM1 to antibiotics was tested on medium containing antibiotics 100 µg mL\(^{-1}\) ampicillin, 10 µg mL\(^{-1}\) erythromycin, 10 µg mL\(^{-1}\) tetracycline, and 20 µg mL\(^{-1}\) kanamycin, respectively. The results of the susceptibility test showed that the bacterial colonies could grow on a medium with antibiotics erythromycin and kanamycin. These results assumed that B. halodurans CM1 has resistant genes against erythromycin and kanamycin.

Bacillus halodurans CM1 is one of the bacterial strains collected by Badan Pengkajian dan Penerapan Teknologi (BPPT) Culture Collection. This B. halodurans CM1 strain is alkalothermophilic bacteria that were previously isolated from hot spring sediment in Cimanggu, West Java. Based on DNA sequence analysis of the 16S rRNA gene, B. halodurans CM1 was identified as a new strain that has 99% similarity with B. halodurans C-125 [13]. Research on the B. halodurans CM1 assessment has not been studied in depth. The potential gene from B. halodurans CM1 can be engineered for further research, such as genes that are resistant to antibiotics. Studies on isolation of the erythromycin-resistant antibiotic genes from B. halodurans CM1 have never been conducted. This study aimed to isolate the erythromycin-resistant gene from B. halodurans CM1 using the PCR approach and cloned into Escherichia coli DH5α using the pGEM-T Easy vector.

2. Methods and Materials

2.1. Bacterial growth and erythromycin Minimum Inhibitory Concentration (MIC) test
Bacillus halodurans CM1 was routinely grown and maintained aerobically on Horikoshi agar/broth at 50 °C. Susceptibility to erythromycin was determined by spotting Horikoshi culture supplemented with different concentrations of erythromycin (ChemCruz, Heidelberg, Germany) and incubated aerobically at 50 °C for overnight. As there are no specific established antibiotic breakpoints values for B. halodurans CM1, the breakpoint values used for categorizing Bacillus sp. as resistant were those recommended by Barbosa et al. [14]. Initial tests were performed with plates supplemented with 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mg mL\(^{-1}\) erythromycin. The concentration range of erythromycin was subsequently expanded, with plates supplemented with 1.0, 1.5, and 2.0 mg mL\(^{-1}\). In this test, B. subtilis was grown on the same agar media to act as a control.

2.2. Medium
Horikoshi medium was used for the cultivation of B. halodurans CM1. Luria Bethani (LB) medium contain ampicillin, X-Gal, and IPTG were used for the screening of recombinant Escherichia coli DH5α.

2.3. Genomic DNA extraction
Extraction of the genome DNA of B. halodurans CM1 performed using Genom DNA Mini Kit [Promega, USA]. The result of extraction genome visualization was observed 1% agarose gel with 100 volts for 28 minutes by electrophoresis.
2.4. Amplification of fragment gene encoding erythromycin rRNA methylase (erm)

The primers were designed based on sequences of gene encoding  
erythromycin rRNA methylase (erm)  
from B. halodurans C-125 on the site http://www.genome.jp. Forward primer 5’-ATGATCGCTA TGACGAAAAAGAAGC-3’ and reverse primer 5’-TTATAATGGTGTTTCTCGGTCGTGG-3’ were used in the amplification of the gene target. The amplification was performed using GoTaq® Flexi DNA polymerase based on the protocol of Promega [15] under PCR condition initial denaturation 95 °C 2 min, followed by 30 cycles of denaturation 95 °C 1 min, annealing 55-58 °C 1 min, and extension 72 °C 2 min, then continued to final extension 72 °C 5 min.

2.5. Transformation of recombinant plasmid pGEM-T Easy to Escherichia coli DH5α

Ligation of the PCR fragment into the pGEM-T easy vector using T4 DNA ligase was carried out based on the protocol of Promega. Plasmid recombinant that contained the PCR product was transformed into competent cell E. coli DH5α by heat shock methods [16]. Screening of transformant was performed by screening blue-white that uses LB agar containing 100 µg ml⁻¹ampicillin, 0.1 M isopropyl β-D-1-thiogalactopyranoside (IPTG), and 4% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). The culture then was incubated over night at 37°C. The positive colonies that contain plasmid recombinants are white color, but the negative colonies are a blue color. Positive colonies were cultured in a liquid LB medium containing ampicillin for extraction plasmid DNA.

2.6. Extraction and verification of recombinant plasmid from Escherichia coli DH5α

Extraction plasmid from positive colonies of recombinant E. coli DH5α was performed by GeneJET Plasmid Miniprep Kit. The extracted plasmid was confirmed by digestion using the HindIII and EcoRI enzyme.

2.7. Sequencing and analysis of DNA sequences

The plasmid that has been confirmed by HindIII and EcoRI was delivered to First BASE sequencing services for sequencing using the universal primer forward M13F-pUC(-40) and primer reverse M13R-pUC(-26). The sequencing result is then analyzed with Snapgene software and aligned using CLUSTAL W program at http://www.genome.jp. Sequencing analysis to investigate the similarity with other genes is conducted using genomic databases available on GenBank and its Local Search Alignment Tool (BLASTn or BLASTx) provided at http://www.ncbi.nlm.nih.gov/blast.cgi. Gene sequences in the form of nucleotide bases were translated into amino acids using the Translate Tool program on https://web.expasy.org/translate/.

3. Results and discussion

B. halodurans CM1 culture had optimal growth incubated on Horikoshi pH 9 at 50 °C. B. halodurans CM1 susceptibility to erythromycin was tested with a minimum inhibitory concentration test (MIC). MIC values are defined as the lowest concentration of an antibiotic that can inhibit the growth of microorganisms after overnight incubation [17]. This MIC test was carried out on agar and broth medium. At the initial MIC test, the colonies could grow well at erythromycin concentration of 0.2, 0.4, 0.6, and 0.8 mg mL⁻¹, but at the concentration 1.0 mg mL⁻¹, few colonies could grow on the medium. The growth of the colonies can be observed in Figure 1. At extended erythromycin concentration, colonies of B. halodurans CM1 could not growth at a concentration of 2.0 mg ml⁻¹, seen with a clear medium as in Figure 2.

Genomic DNA could be extracted from B. halodurans CM1, and the results were visualized on a 1% agarose gel, and fragment more than 10,000 bp was detected as shown in Figure 3. The genome can be extracted well and did not contain any contaminants. By using the designed primers, the target gene was amplified using GoTaq® Flexi DNA polymerase based on the protocol of Promega. The annealing temperature used in the PCR reaction was 56 °C for 1 minute. The target gene was visualized using 1.5 % agarose gel with 100 volts for 28 minutes. The specific bands from the target gene lies between 750 bp and 1.000 bp were detected as shown in Figure 4. The target gene has a size of around 864 bp.
In this study, pGEM T-easy vector was used, since this cloning was a TA cloning vector that utilized the PCR product by Taq polymerase that had A-cohesive end, blue-white screening system, T7 or SP6 promoter system, and gave very good result in gene isolation in many reports [18, 19, 20].

**Figure 1.** MIC tests on *B. halodurans* CM1 growth on Horikoshi pH 9 agar at 50°C with various erythromycin concentration. A) Horikoshi (without erythromycin); B) Horikoshi (+0.2 mg mL⁻¹ erythromycin); C) Horikoshi (+0.4 mg mL⁻¹ erythromycin); D) Horikoshi (+0.6 mg mL⁻¹ erythromycin); E) Horikoshi (+0.8 mg mL⁻¹ erythromycin); and F) Horikoshi (+1.0 mg mL⁻¹ erythromycin).

**Figure 2.** MIC tests on *B. halodurans* CM1 growth on Horikoshi pH 9 agar at 50°C with various erythromycin concentration. A) Horikoshi (+0.5 mg mL⁻¹ erythromycin); B) Horikoshi (+1.0 mg mL⁻¹ erythromycin); C) Horikoshi (+1.5 mg mL⁻¹ erythromycin); and D) Horikoshi (+2.0 mg mL⁻¹ erythromycin).

**Figure 3.** Extraction of *B. halodurans* CM1. Lane: M) GeneRuler 1 kb DNA ladder and 1) DNA genomic of CM1.

**Figure 4.** Amplification of the target gene from *B. halodurans* CM1. Lane: M) GeneRuler 1 kb DNA ladder; 1) negative control; and 2) target gene from CM1.

After ligation of the PCR product into the pGEM-T Easy vector, the white colonies grew on transformation plate were picked. White colonies were thought to contain target gene fragment (*ErmK* genes), while blue colonies were thought to contain colonies that do not contain parent gene fragments. The formation of these white and blue colonies is due to the selection of markers in the pGEM-T Easy vector. The vector has a selection marker in the form of an ampicillin resistance gene and an additional selection marker in the form of the *LacZ* gene. The carrier gene which is resistant to

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**Note:** The images and text are aligned to match the original layout of the document. The diagrams and figures are represented as described in the text. The text is written in a clear and logical manner, ensuring all necessary information is conveyed accurately and coherently.
ampicillin encodes the β-Lactamase enzyme. The β-Lactamase enzyme will degrade ampicillin so that when recombinant plasmid-carrying cells were grown in media containing ampicillin, the cells would be grown while cells that did not carry recombinant plasmids would have died. The Lac Z gene encodes the galactosidase enzyme. This enzyme would be hydrolyzed X-Gal which was added to the selection medium into galactoside and its derivative compounds, 5-bromo-4-chloro indoxyl which were blue. When there was a gene fragment inserted in the plasmid, the synthesis of α-peptide which acted as an activator for the action of the enzyme β-Galactosidase would be inhibited so that the blue color was not formed [21, 22].

![Diagram](image_url)

**Figure 5.** A. Extraction of recombinant plasmid pGEM.**Erm**CM1-ORF. Lane: M) GeneRuler 1 kb DNA ladder; 1) K4; 2) K12; 3) K17; 4) K20; 5) K31; and 6) K42. B. Digestion of recombinant plasmid pGEM.**Erm**CM1-ORF by **Hind**III (lane 1-6) and **Eco**RI (lane 7-9). Lane: M) GeneRuler 1 kb DNA ladder; 1) K4; 2) K12; 3) K17; 4) K20; 5) K31; 6) K42; 7) K4; 8) K17; and 9) K20.

There are six white colonies that grow on LB pH 7 medium containing ampicillins. The six colonies were assumed to contain recombinant plasmids and then extracted and visualized in 1% agarose gel with 100 volts for 28 minutes in Figure 5A. The recombinant plasmid has backbone with a size 3015 bp and 864 bp of the target gene. Recombinant plasmid verification uses the **Hind**III and **Eco**RI restriction enzyme. **Hind**III restriction enzyme has cut off one side so the plasmid becomes linear with a size of around 3883 bp in Figure 5B (lane 1-6), however the **Eco**RI restriction enzyme has cut off two sides so that there are two bands with sizes around 3015 bp and 864 bp in Figure 5B (lane 7-9). Verification with **Hind**III and **Eco**RI was carried out to confirm that the recombinant
plasmid carried the *erm* gene insert. The clones that were confirmed by HindIII and EcoRI digestion were further used for sequencing. Two out of the six clones from the verification results were sequenced to First BASE sequencing services using universal primers.

| ErmKC125-ORF  | ATGATCGCTATGAGAAGAAAGAGCTAATATTCA |
|---------------|-----------------------------------|
| ErmKC1-ORF_M13RC | GCCATTCGCCGCGCAGGAAATGATGATGAGAAGAAAGAGCTAATATTCA |
| ErmKC1-ORF_M13F  | GCCATTCGCCGCGCAGGAAATGATGATGAGAAGAAAGAGCTAATATTCA |
| ErmKC125-ORF  | GCCATTCGCCGCGCAGGAAATGATGATGAGAAGAAAGAGCTAATATTCA |
| ErmKC1-ORF_M13RC | GCCATTCGCCGCGCAGGAAATGATGATGAGAAGAAAGAGCTAATATTCA |
| ErmKC1-ORF_M13F  | GCCATTCGCCGCGCAGGAAATGATGATGAGAAGAAAGAGCTAATATTCA |
| ErmKC125-ORF  | ATAAAAGGCATCTGCGCACAGAAATTGCGACGAGGAAAGTTACTAAGATACCCGTA |
| ErmKC1-ORF_M13RC | ATAAAAGGCATCTGCGCACAGAAATTGCGACGAGGAAAGTTACTAAGATACCCGTA |
| ErmKC1-ORF_M13F  | ATAAAAGGCATCTGCGCACAGAAATTGCGACGAGGAAAGTTACTAAGATACCCGTA |
| ErmKC125-ORF  | TAGAGCTCCGTGCCTGCGTCAGCAAAGGCGCTTGAGACGAGGTTAGTTTTATAGGAGCAAGGAGAAAGAG |
| ErmKC1-ORF_M13RC | TAGAGCTCCGTGCCTGCGTCAGCAAAGGCGCTTGAGACGAGGTTAGTTTTATAGGAGCAAGGAGAAAGAG |
| ErmKC1-ORF_M13F  | TAGAGCTCCGTGCCTGCGTCAGCAAAGGCGCTTGAGACGAGGTTAGTTTTATAGGAGCAAGGAGAAAGAG |
| ErmKC125-ORF  | TGCTCGCATGCGAATGACCAAATCGTTATCAAGTTAAAAACGGAAGATGCGGACG |
| ErmKC1-ORF_M13RC | TGCTCGCATGCGAATGACCAAATCGTTATCAAGTTAAAAACGGAAGATGCGGACG |
| ErmKC1-ORF_M13F  | TGCTCGCATGCGAATGACCAAATCGTTATCAAGTTAAAAACGGAAGATGCGGACG |
| ErmKC125-ORF  | CCGCCATACAGCAGACAGATCAGTACACAGGAGCATTAGGAGCATTACCTGATAAGGGGAAT |
| ErmKC1-ORF_M13RC | CCGCCATACAGCAGACAGATCAGTACACAGGAGCATTAGGAGCATTACCTGATAAGGGGAAT |
| ErmKC1-ORF_M13F  | CCGCCATACAGCAGACAGATCAGTACACAGGAGCATTAGGAGCATTACCTGATAAGGGGAAT |
| ErmKC125-ORF  | TGTTGCCTGGTTTCCAAATTACTCCATATGCGATAAGCTATAGGAATAGGCTCTTT |
| ErmKC1-ORF_M13RC | TGTTGCCTGGTTTCCAAATTACTCCATATGCGATAAGCTATAGGAATAGGCTCTTT |
| ErmKC1-ORF_M13F  | TGTTGCCTGGTTTCCAAATTACTCCATATGCGATAAGCTATAGGAATAGGCTCTTT |
| ErmKC125-ORF  | CCATTCGGATGCTGGTTTACAGGAGGATGCTGATGATGAAAAAGAGAGGACGAGGACGAC |
| ErmKC1-ORF_M13RC | CCATTCGGATGCTGGTTTACAGGAGGATGCTGATGATGAAAAAGAGAGGACGAGGACGAC |
| ErmKC1-ORF_M13F  | CCATTCGGATGCTGGTTTACAGGAGGATGCTGATGATGAAAAAGAGAGGACGAGGACGAC |
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Figure 6. The nucleotide alignment sequence of ErmK gene Bacillus halodurans CM1 compared to B. halodurans C-125.

The DNA sequencing results of the two clones showed that both clones showed an open reading frame of protein as shown in Figure 7. Analyze of DNA sequence with BLAST showed has 99% similarity with gene encoding erythromycin resistance from B. halodurans C-125 (GenBank No: BA000004.3, ErmK). The ErmK gene from B. halodurans CM1 has the same size of 864 bp as in B. halodurans C-125 [23]. Based on the results of nucleotide alignment, there are three different nucleotides compared to B. halodurans C-125. The three nucleotides are thymine at 464th and 492nd orders and guanine at 713rd orders as shown in Figure 6.

The ErmK gene nucleotide sequence is translated using the ExPASy Translate Program. The gene encoded 288 deduced amino acid as shown in Figure 7. Based on the analysis of amino acid sequences, there is a different one amino acid that encodes leucine in 155th orders. This pattern supports the research conducted by Ulfah et al. [13] that B. halodurans CM1 is a new bacterial strain compared to B. halodurans C-125.
4. Conclusion
It is concluded that *B. halodurans* CM1 colonies have a susceptibility to erythromycin at a concentration of 1 mg mL\(^{-1}\). The size of the *ErmK* gene is 864 bp that encodes 288 amino acids. The gene had 99% similarity with gene encoding erythromycin resistance from *B. halodurans* C-125 (GenBank No access: BA000004.3, *ErmK*). Based on the analysis, there are 3 nucleotides in the *ErmK* sequence of *B. halodurans* CM1 that are different from that of another well-known strain *B. halodurans* C-125. This dissimilar pattern of sequence encodes Leucine instead of Serine in 155\(^{th}\) orders of CM1 amino acid sequence. The results of gene isolation and sequencing will be used for research analyzing erythromycin-resistant functional gene product from *B. halodurans* CM1.

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**Figure 7.** The alignment of deduced amino acids of *ErmK* gene *B. halodurans* CM1 compared to *B. halodurans* C-125.
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